09:42:06 OCA PAD INITIATION - PROJECT HEADER INFORMATION 08/17/89

Active

Project #: G-33-U08 Cost share #: Rev #: 0
Center # : 10/24-6-Q5270-8A0 Center shr #: OCA file #:
Work type : RES
Contract#: 5 R01 HL29307-08 Mod #: Document : GRANT
Prime #: Contract entity: GIT

Subprojects ? : N
Main project #:

Project unit: CHEM Unit code: 02.010.136
Project director(s):
POWERS J C CHEM (404)894-4038

Sponsor/division names: DHHS/PHS/NIH / NATL INSTITUTES OF HEALTH
Sponsor/division codes: 108 / 001

Award period: 890815 to 900731 (performance) 901031 (reports)

| Sponsor amount | New this change | Total to date |
|---|---|---|
| Contract value | 158,006.00 | 158,006.00 |
| Funded | 158,006.00 | 158,006.00 |
| Cost sharing amount | | 0.00 |

Does subcontracting plan apply ?: N

Title: SYNTHETIC ELASTASE INHIBITORS

PROJECT ADMINISTRATION DATA

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Security class (U,C,S,TS) : U
Defense priority rating : N/A
Equipment title vests with: Sponsor

ONR resident rep. is ACO (Y/N): N
NIH supplemental sheet
GIT X


Administrative comments -
INITIATION OF 8TH YEAR OF PROJECT. CONTINUATION OF G-33-U07.

GEORGIA INSTITUTE OF TECHNOLOGY
OFFICE OF CONTRACT ADMINISTRATION

NOTICE OF PROJECT CLOSEOUT

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Closeout Notice Date 09/11/91

Project No. G-33-U08_____ Center No. 10/24-6-Q5270-8A0_

Project Director POWERS J C_____ School/Lab CHEMISTRY_____

Sponsor DHHS/PHS/NIH/NATL INSTITUTES OF HEALTH_____

Contract/Grant No. 5 R01 HL29307-08_____ Contract Entity GIT_

Prime Contract No. _____

Title SYNTHETIC ELASTASE INHIBITORS_____

Effective Completion Date 910731 (Performance) 911031 (Reports)

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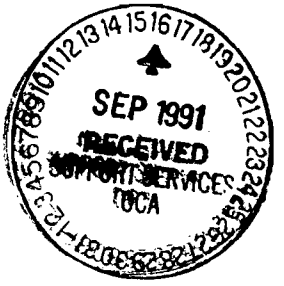
| Closeout Actions Required: | Y/N | Date Submitted |
|---|---|---|
| Final Invoice or Copy of Final Invoice | Y | _____ |
| Final Report of Inventions and/or Subcontracts | Y | _____ |
| Government Property Inventory & Related Certificate | N | _____ |
| Classified Material Certificate | N | _____ |
| Release and Assignment | N | _____ |
| Other _____ | N | _____ |

Comments_____

_____

Subproject Under Mein Project No. _____

Continues Project No. _____




Distribution Required:

| | |
|---|---|
| Project Director | Y |
| Administrative Network Representative | Y |
| GTRI Accounting/Grants and Contracts | Y |
| Procurement/Supply Services | Y |
| Research Property Managment | Y |
| Research Security Services | N |
| Reports Coordinator (OCA) | Y |
| GTRC | N |
| Project File | Y |
| Other _____ | N |
| _____ | N |

NOTE: Final Patent Questionneire sent to PDPI.

G-22-U

# Synthetic Elastase Inhibitors (HL29307)
## Annual Report (April 1, 1990-March 31, 1991)

James C. Powers, PI
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**Specific Aims.** The major specific aim of our proposed research was the development of a synthetic elastase inhibitor which would be useful for the treatment of human emphysema. A variety of inhibitor structures were proposed including heterocyclic mechanism-based inhibitors and peptide transition state analogs. We proposed to test the specificity of all new inhibitors with other PMN proteases such as human leukocyte cathepsin G. A secondary goal of the research was the extension of potent inhibitor structures to other serine proteases. We suggested that inhibitor studies would lead to a better understanding of the active site structures of the serine proteases involved in connective tissue turnover, might produce clinically useful drugs for the treatment of emphysema and related diseases, would stimulate the research of medicinal chemists in pharmaceutical companies, and will provide new tools for the *in vivo* and *in vitro* study of the role of neutrophil and mast cell proteases in a variety of physiological processes.

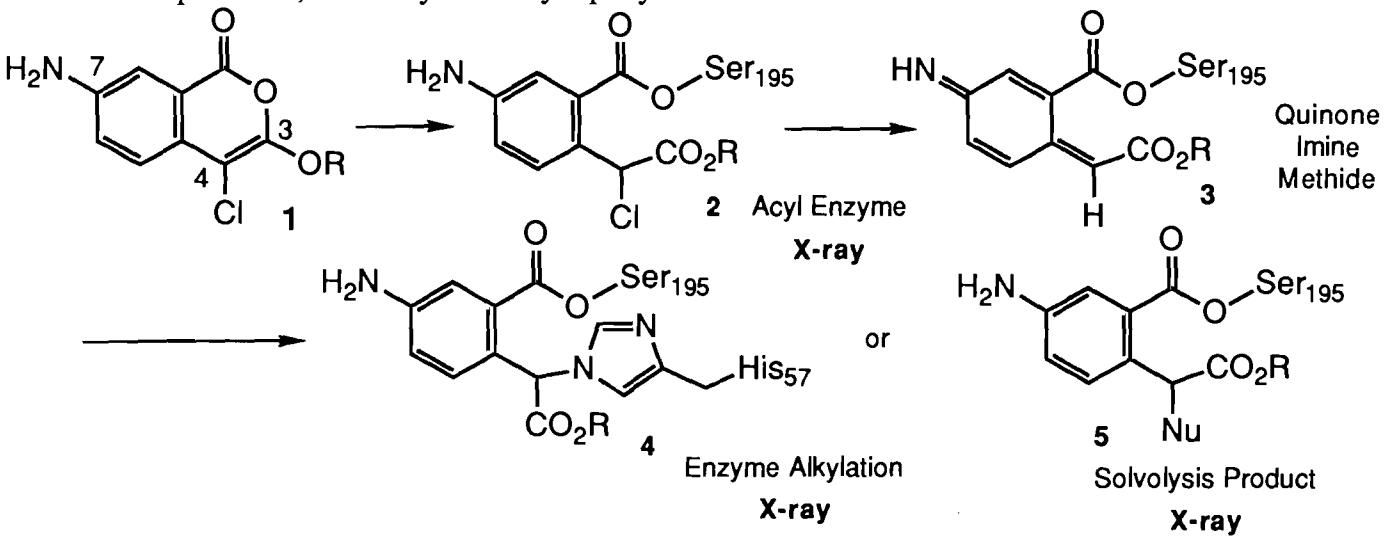
**Summary of progress.** During the 5 year period of this research project, we have developed several new classes of inhibitors for HNE including isocoumarins (mechanism-based inhibitors), peptide α-ketoesters (transition-state analogs), and peptide phosphonates (irreversible transition-state inhibitors). X-ray structural studies have been accomplished with 5 isocoumarin inhibitors bound to the active sites of PP elastase and trypsin, with 4 chloromethyl ketone inhibitors bound to the active site of HNE, and with one peptide α-ketoester inhibitor bound to the active site of PP elastase. The structural studies have led to a better understanding of the active site structures of these two important elastases and their mechanisms of inhibition. Several of the isocoumarin inhibitors developed have been studied with other physiologically important serine proteases and an influenza viral serine esterase. One of our inhibitors, 3,4-dichloroisocoumarin, is now commercially available from four companies for use as a general serine protease inhibitor. Isocoumarin and phosphonate inhibitors may prove to be valuable in other disease states related to serine protease activity and are currently being tested in animal models of sepsis and Alzheimer's disease.

The various classes of elastase inhibitors discovered in my laboratory have been used extensively by pharmaceutical companies in the development of therapeutics for the treatment of emphysema. In particular, the chloromethyl ketone inhibitor MeO-Suc-Ala-Ala-Pro-Val-$CH_2Cl$ is used as the standard elastase inhibitor for the validation of new animal models of emphysema and as a standard elastase inhibitor to compare the effectiveness of newly developed inhibitors (Fletcher et al., 1990). Benzoxazinones and peptide α-ketoesters elastase inhibitors, discovered in my laboratory, have been chosen by Syntex Canada (Krantz et al., 1987; 1990) and Merrill Dow (Peet et al., 1990) for extensive development. Peptide sequences which we discovered to be potent elastase inhibitors have been translated into the sequences found in the potent trifluoromethyl ketone inhibitors of HN elastase which are now undergoing development by ICI Americas as therapeutics (Trainor, 1987). The trifluoromethyl ketone class of elastase inhibitors is probably the closest to reaching clinical use of any of the low molecular weight inhibitors available today and at least one derivative is undergoing phase I testing.

**Isocoumarin Mechanism-Based Inhibitors.** Isocoumarins are mechanism-based (or suicide) heterocyclic serine proteases which are rich in possible masked reactive functional groups. Thus far, we have described isocoumarins which contain latent acid chloride or quinone imine methide functional groups. We have synthesized well over a hundred isocoumarin inhibitors, have published ca. 13 papers and reviews which describe these inhibitors (see publication list at end of this section) and have still have not yet reported the majority of the compounds which we have synthesized. The recent completion of several x-ray structures of complexes of PPE inhibited by isocoumarins has yielded important insights into the binding modes of these inhibitors and we are working on two additional x-ray papers and a J. Med. Chem. paper. In addition to elastase, the isocoumarins have proved to be useful in

the study of an influenza viral esterase and a group of serine proteases isolated from natural killer cells (publication 4, 8, 13, 14).

The inactivation mechanism of serine proteases by 3-alkoxy-7-amino-4-chloroisocoumarins (**1**) is shown below. The active site Ser-195 attacks the isocoumarin carbonyl group and opens the isocoumarin ring to form an acyl enzyme (**2**). This reaction unmasks a latent quinone imine methide functional group (**3**) which is formed by the elimination of HCl from the acyl enzyme. This can react either with an enzyme nucleophile (His-57) to give an irreversibly inhibited enzyme structure (**4**) or with a solvent nucleophile to give a stable acyl enzyme (**5**). Partial reactivation by hydroxylamine with some inhibited derivatives suggests a partitioning between the two enzyme-inhibitor complexes (**4** & **5**) in solution with the nonreactivatable complex (**4**) containing an alkylated histidine residue. Both the 7-amino and 4-chloro groups are required for formation of a stable inactivated enzyme; isocoumarins which lack these features inhibit serine proteases, but deacylate fairly rapidly.




In addition to the kinetic evidence, the proposed inhibition mechanism is supported by x-ray crystallographic studies. Five enzyme-isocoumarin complexes have completed in collaboration with Ed Meyer (Texas A & M) and Wolfram Bode (Max Planck Institute, Munich) and are listed below.

7-amino-4-chloro-3-methoxyisocoumarin & PPE - solvolysis product **5** with Nu = acetate - (structure published, Meyer et al., 1987).

4-chloro-3-ethoxy-7-guanidinoisocoumarin & PPE - acyl enzyme **2** with chlorine still present - structure published in 1990, publication 2.

4-chloro-3-ethoxy-7-guanidinoisocoumarin & trypsin - mixture of acyl enzyme **2** and the histidine alkylation product **4** - structure published in J. Am. Chem. Soc.

7-amino-3-bromoethoxy-4-chloroisocoumarin & PPE - histidine alkylation product **4** - structure published.

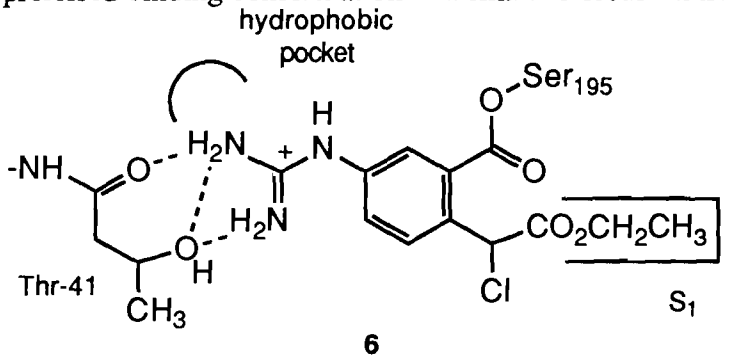
7-(Tos-Phe-NH)-4-chloro-3-ethoxyisocoumarin & PPE - histidine alkylation product **4** - structure complete and will be submitted soon.

All the major products (**2**, **4**, and **5**) in the reaction scheme have been observed crystallographically with different isocoumarins. The observation of the doubly covalent bond histidine alkylation product **4** is the second time such a derivative has been observed with a serine protease. The first doubly covalently bound adduct was observed in a crystal structure of PPE inhibited by a β-lactam inhibitor which was reported by a Merck group (Navia et al., 1987). The exact product formed in the inhibition reaction varies with both the enzyme and the isocoumarin inhibitor. Different inhibitors give variable ratios of hydroxylamine reactivatable acyl enzymes (**2** or **5**) or non-reactivatable alkylation products (**4**). The solvolysis product **5** is probably an artifact of that particular crystallographic experiment which was carried out in an acetate buffer at pH 5.0, a pH where the His-57 would be

protonated and less likely to undergo alkylation. Kinetic studies with a variety of inhibitors indicate that histidine alkylation is usually the favored product at neutral pH with both elastases and most of the isocoumarins studied.

**Isocoumarin Binding Modes**. The binding modes of all five isocoumarins complexed to PPE and trypsin are remarkably different even though all are tethered to Ser-195 via an ester bond. The carbonyl group of the ester bond linking Ser-195 to the inhibitor is in the oxyanion hole of the serine protease in the 7-amino-4-chloro-3-methoxyisocoumarin complex with PPE, while it is twisted out of the oxyanion hole in the 7-guanidino structure. The twisting of the ester carbonyl group allows favorable hydrogen bonding between the 7-guanidino group and Thr-41 (**6**) and explains the stability of the complex toward deacylation. His-57 is either hydrogen bonded to the ester bond or is covalently linked with the inhibitor (**4**).

Schematic drawings of the interactions observed in the PPE complexes with 4-chloro-3-ethoxy-7-guanidinoisocoumarin (**6**) and the 3-bromoethoxyisocoumarin (**7**) are shown below and a stereo drawing of 3 heterocyclic inhibitors superimposed in the active site of PPE is shown in Fig. 11 of publication 9. In the four PPE complexes, the 3-alkoxy group of the isocoumarin is either in the $S_1$ pocket or lying nearby. However in the complex of trypsin with 4-chloro-3-ethoxy-7-guanidinoisocoumarin, the 7-guanidino group of the isocoumarin is inserted into the $S_1$ pocket of trypsin to interact with Asp-189 in trypsin's binding pocket, while the 3-alkoxy group points toward the S' subsites of the enzyme. This orientation is almost 180° from the orientation found in the complex of the same compound with PPE (**6**). Thus a significantly different binding geometry has been observed for the first time when the same inhibitor is examined with different two serine proteases. It is clear that we don't know all the rules for predicting the preferred binding conformation of a small molecule inhibitor to the active site of a serine protease.

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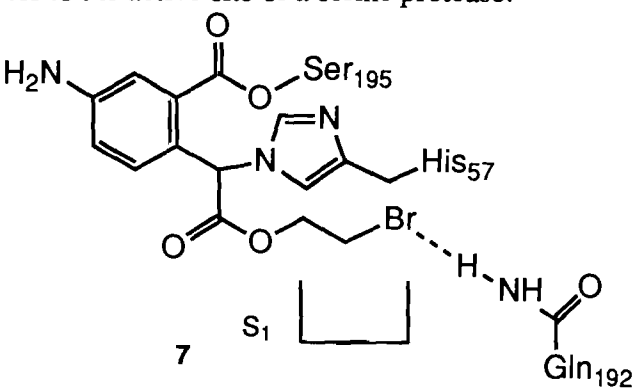


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**Molecular Modeling and Inhibitor Design.** Although it is not yet possible to predict the binding mode of a new isocoumarin inhibitor to elastase, molecular modeling with x-ray crystal structures is extremely useful for improving that inhibitor and for interpreting inhibition kinetic data obtained with related inhibitor structures. For example, molecular modeling of the 7-guanidinoisocoumarin PPE complex **6** suggested that the addition of a small alkyl group (*t*-butyl) to the guanidino group might increase affinity to the enzyme due to the presence of a small hydrophobic pocket near the terminal nitrogen of guanidino group and above Thr-41. Therefore, we synthesized a series of 7-alkyl-NH-CO-NH- derivatives of 4-chloro-3-ethoxyisocoumarin. These ureas were chosen for synthesis due to the difficulty of synthesizing alkyl guanidino derivatives. Replacement of the 7-guanidino group by a urea functional group resulted in almost no loss of inhibitory potency (see following table). As predicted, the *t*-butyl-NH-CO-NH- derivative was the most effective PPE inhibitor and had a second order inhibition rate $k_{obsd}/[I]$ which was 3.5 fold higher than the parent inhibitor. Indeed, the compounds developed from the modeling are among the best irreversible inhibitors reported thus far for PPE (work is described in publication 2). In order to confirm the binding mode predicted from molecular modeling, the Meyer group is currently determining the structure of PPE inhibited by 7-(*t*-Bu-HN-CO-NH)-EtOIC.

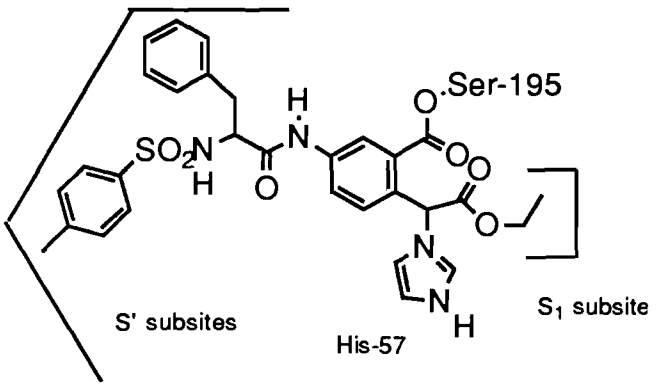
| | | |
|---|---|---|
| 7-($H_2N$-C(=$NH_2^+$)-NH)-EtOIC | 2300 $M^{-1}s^{-1}$ | |
| 7-($H_2N$-CO-NH)-EtOIC | 2200 | |
| 7-(Me-HN-CO-NH)-EtOIC | 1400 | |
| 7-(Et-HN-CO-NH)-EtOIC | 1700 | EtOIC = |
| 7-(*i*-Pr-HN-CO-NH)-EtOIC | 4900 | 4-chloro- |
| 7-(*t*-Bu-HN-CO-NH)-EtOIC | **8100** | 3-ethoxy |
| 7-(Ph-HN-CO-NH)-EtOIC | 4200 | isocoumarin |

One we realized that the 3-alkoxy group of the isocoumarin was fitting into the $S_1$ pocket of elastase, we synthesized a number of derivatives with 3-alkoxy groups of varying length. With HNE, we found the following inhibition constants: MeO, 10,000; EtO, 9,400; PrO, 54,000; and bromoethoxy, 200,000. Clearly HNE prefers the long alkoxy groups. The structure determination of the complex of the 3-bromoethoxyisocoumarin (**7**) with PPE has allowed us to interpret our kinetic data with PPE (see following table, all the bromoethoxy derivatives are ca. 100 fold better with HNE, data not shown). First it is clear that the bromopropoxy derivatives with a slightly long alkoxy group are over 100 fold poorer inhibitors of PPE and HNE. All of the derivatives in which a hydrophobic group is placed on the 7-amino group are 3-7 fold better inhibitors than the parent with the exception of the phenyl urea derivative which is much poorer. The 7-amino group of the isocoumarin points toward the S' subsites of PPE and HNE and we predict that the hydrophobic groups in the extended inhibitors are interacting with the $S_2'$ subsite (Leu-143, Leu-151, Thr-41 in PPE). The phenyl urea derivative is probably so rigid, that the 7-substituent can't twist and adopt a favorable conformation in the active site.

| 7-substituted-4-chloro-3-(2-bromoethoxy)isocoumarin | |
|---|---|
| 7-$NH_2$ | 1,000 $M^{-1}s^{-1}$ |
| 7-(*t*-Bu-NH-CO-NH) | 6,600 |
| 7-(Ph-NH-CO-NH) | 36 |
| 7-(Ph-$CH_2$-NH-CO-NH) | 3,010 |
| 7-(R-($C_6H_5$)($CH_3$)CH-NH-CO-NH) | 9,900 |
| 7-(Ph-$CH_2$-CO-NH) | 4,950 |
| 7-substituted-4-chloro-3-(3-bromopropoxy)isocoumarin | |
| 7-$NH_2$ | 10 |
| 7-(Ph-$CH_2$-NH-CO-NH) | 13 |
| 7-(Ph-$CH_2$-CO-NH) | 28 |

**Human Neutrophil Elastase Inhibitors.** Unfortunately, no crystal structures of an isocoumarin bound to HNE is available or likely to become available. Wolfram Bode and the Merck group have tried extensively to crystallize complexes of HNE bound to small molecule inhibitors. Up to the present, the smallest inhibitor which has yielded crystals with HNE is a tetrapeptide such as MeO-Suc-Ala-Ala-Pro-Val-$CH_2$Cl. However, the crystal structures obtained with PPE have been invaluable for modeling with HNE. The active site structures of PPE and HNE have many features in common (see the review, publication 9) and so we feel quite comfortable using the PPE crystal structures for modeling with HNE. For example, the complex of the 7-Tos-Phe derivative of 4-chloro-3-ethoxyisocoumarin has been determined and has the 3-alkoxy group in the $S_1$ pocket and the Tos-Phe group interacting with the S' subsites of PPE (shown below left). Representative inhibition rates with HNE are shown below (right). The derivatives shown all have a 3-propoxy group instead of the 3-ethoxy group shown in the crystal structure since we demonstrate that HNE preferred to an alkoxy group with one more methylene group. Clearly, most of the rates are so fast that the rate constants should be considered to be lower limits.

The active site of HNE is much more hydrophobic than that of PPE. Two significant changes are: Thr-41 in PPE is replaced in HNE by Phe-41 and Gln-192 is changed to Phe-192. Molecular modeling with HNE indicates that the Tos-Phe group is interacting with the S' subsites consisting of the side chains of Phe-41, Leu-35, and Leu-143. Clearly all the hydrophobic 7-substituents are making favorable contacts with HNE and as a result are extremely potent inhibitors.




| | |
|---|---|
| Tos-Phe | 33,400 $M^{-1}s^{-1}$ |
| $CH_3CO$ | >190,000 |
| $CH_3CH_2CH_2CO$ | >124,000 |
| $(CH3)_2CHCH_2CO$ | >220,000 |
| $PhCH_2CH_2CO$ | >250,000 |
| $CH_3SCH_2CO$ | >152,000 |
| $3\text{-}O_2N\text{-}C_6H_4CO$ | >210,000 |
| $CH_3CH_2OCO$ | >181,000 |
| $CH_3CH_2HNCO$ | >276,000 |
| PhNHCO | 143,000 |
| $PhCH_2NHCS$ | >131,000 |
| Ph-HNCS | >166,000 |

**Dichloroisocoumarin.** We should also mention 3,4-dichloroisocoumarin which we reported as a general serine protease inhibitor in 1985 (publication 29). HLE and PPE are both inhibited effectively by 3,4-dichloroisocoumarin with $k_{obsd}/[I]$ values of 8900 and 2500 $M^{-1}s^{-1}$ respectively. Although the elastases are most effectively inhibited, dichloroisocoumarin is a general serine protease inhibitor and reacts with all serine proteases which have been investigated thus far. It is now commercially available from four companies and is becoming widely used for diagnosing new serine proteases since it is more reactive than PMSF and less hazardous than DFP. It addition it has also been invaluable to show that serine proteases are involved in natural killer cell and cytotoxic T lymphocyte granule mediated killing of targeted cells (publications 4, 13, 14). I expect to see this inhibitor increasingly used by other investigators in the future.

**Peptide Phosphonate Inhibitors**. Peptidyl derivatives of α-aminoalkylphosphonate diphenyl ester are effective and specific inhibitors of serine proteases at low concentrations. These inhibitors are chemically stable, relatively easy to synthesize, and do not react with acetylcholinesterase. Previously synthesized phosphonate inhibitors of serine proteases include phosphonyl fluorides or phosphonamides which are unstable in solution and/or reactive toward acetylcholinesterase. Our phosphonate diphenyl esters form very stable derivatives with serine proteases due to the resemblance between the inhibition product (phosphonyl derivative shown below) and the tetrahedral intermediate involved in peptide bond hydrolysis. Our phosphonates are stable for at least 3 days in human plasma and the inhibited enzyme has a half-life for reactivation of 10 hrs in the case of chymotrypsin and >3 days in the case of the elastases. Although phosphonates are not as reactive as isocoumarins, we believe that they have considerable utility as therapeutic agents due their high stability and specificity.


Aminoalkylphosphonate diphenyl esters are very specific for the target serine protease and the data suggests that good interactions with the $S_1$ pocket of the target serine protease are necessary before nucleophilic substitution on phosphorus atom occurs to give a stable phosphonyl derivative. For example, Suc-Val-Pro-$Phe^P(OPh)_2$ reacts with chymotrypsin ($k_{obsd}/[I]$ = 44,000 $M^{-1}s^{-1}$) and does not react with elastases. Boc-Val-Pro-$Val^P(OPh)_2$ reacts with HNE (27,000 $M^{-1}s^{-1}$) and PPE (11,000 $M^{-1}s^{-1}$) and does not react with chymotrypsin. The longer peptides with a C-terminal phosphonate related to

phenylalanine are also inhibitors for other chymotrypsin-like enzymes such as rat mast cell protease II and human cathepsin G.

| | ChyT | PPE | HNE |
|---|---|---|---|
| MeO-Suc-Ala-Ala-Pro-Nva$^{P}$(OPh)$_2$ | 50 | 4200 | 380 |
| MeO-Suc-Ala-Ala-Ala-Val$^{P}$(OPh)$_2$ | 15 | 2800 | 1500 |
| MeO-Suc-Ala-Ala-Ala-Phe$^{P}$(OPh)$_2$ | 2,000 | NI | NI |
| MeO-Suc-Ala-Ala-Pro-Val$^{P}$(OPh)$_2$ | 21 | 7,100 | 7,100 |
| MeO-Suc-Ala-Ala-Pro-Leu$^{P}$(OPh)$_2$ | 1,500 | 740 | 140 |
| MeO-Suc-Ala-Ala-Pro-Phe$^{P}$(OPh)$_2$ | 11,000 | NI | NI |
| MeO-Suc-Ala-Ala-Pro-Met$^{P}$(OPh)$_2$ | 570 | 44 | 53 |
| MeO-Suc-Ala-Ala-Pro-Met(O)$^{P}$(OPh)$_2$ | 15 | 1.6 | 1.6 |
| Boc-Val-Pro-Val$^{P}$(OPh)$_2$ | NI | 11,000 | 27,000 |
| Z-Phe-Pro-Phe$^{P}$(OPh)$_2$ | 17,000 | NI | NI |
| Suc-Val-Pro-Phe$^{P}$(OPh)$_2$ | 44,000 | NI | NI |

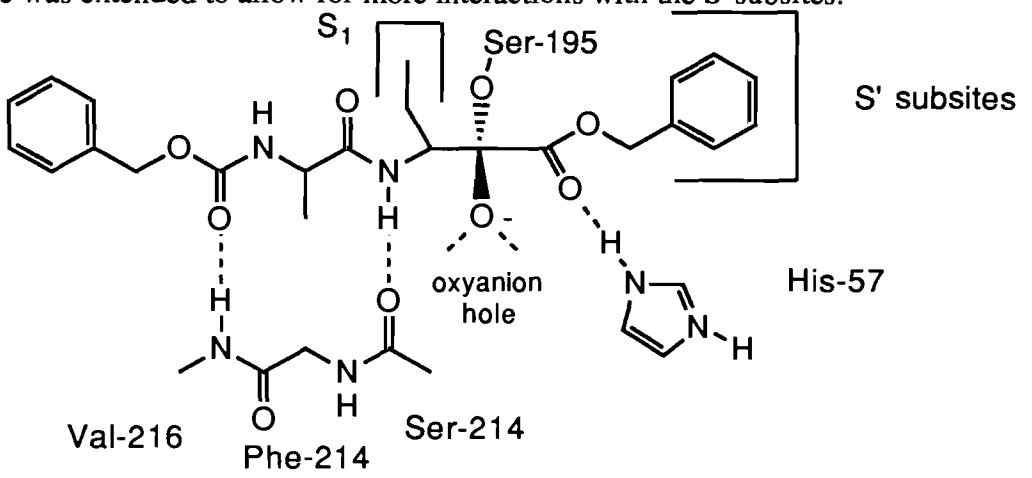
The inhibition reaction is stereospecific since $^{31}$P NMR studies have shown than only one of the two stereoisomers of Suc-Val-Pro-Phe$^{P}$(OPh)$_2$ reacts with chymotrypsin with a rate constant which is higher than (146,000 $M^{-1}s^{-1}$) the mixture. The $^{31}$P NMR of chymotrypsin inhibited by this peptide shows one broad signal at 25.98 ppm corresponding to the Ser-195 phosphonate ester which is consistent with the product structure shown above.

**Peptide α-Ketoester Transition-State Inhibitors.** A variety of transition-state inhibitors for elastase are currently available including peptide aldehydes, peptide boronic acids, and peptide trifluoromethyl ketones. During the course of this research, we developed novel peptide α-ketoesters as another type of transition-state inhibitor for elastases. This class of inhibitors was based on the refined x-ray crystal structure of the complex formed between bovine trypsin and the ketoacid inhibitor 4-amidinophenylpyruvate (APPA), a potent trypsin inhibitor (Walter and Bode, 1983). In the x-ray structure, the amidinophenyl group is located in the primary specificity pocket of trypsin and the active site serine has added to the 2-carbonyl group in APPA. The oxyanion is stabilized by hydrogen bonding with groups in the oxyanion hole of trypsin. A unique feature of this structure is the hydrogen bonding observed between the carboxylate oxygen and the serine oxygen and the NH of histidine-57. We expected that the negative charge on the carboxylate would significantly contribute to the binding energy of α-ketoacids.

We designed and synthesized a number of peptide derivatives of various α-ketoacids and α-ketoesters derived from several different amino acids. Some of our kinetic results are shown in the following table where $K_I$ values are report in μM in 0.1 M Hepes, pH 7.5, 0.5 M NaCl, 9.0-9.8% $Me_2SO$ at 25°C (Ala-$CO_2$Et = -NHCH($CH_3$)CO-$CO_2$Et; Abu = 2-aminobutanoic acid). Surprisingly, the α-ketoesters are much better inhibitors than the corresponding acids which indicates that additional interactions with the $S_1'$ subsite and hydrogen bonding of the ester oxygen with the histidine N-H can result in significant binding energy in the case of elastase. The best inhibitors, Z-Ala-Ala-DL-Abu-COOEt, the corresponding benzyl ester, and Z-Ala-Ala-Ala-DL-Ala-COOEt, had $K_I$ values in the submicromolar range (publication 28). Importantly, MeO-Suc-Val-Pro-Phe-COOMe is one of the most potent reversible cathepsin G inhibitors known.

| Inhibitor | HLE | PPE | Cat G |
|---|---|---|---|
| Bz-DL-Ala-COOEt | 640 μM | 590 | |
| Bz-DL-Ala-COOH | 3100 | 3200 | |
| Z-Ala-Ala-DL-Abu-COOEt | 0.12 | 0.15 | |
| Z-Ala-Ala-DL-Abu-COOBzl | 0.09 | 0.08 | |
| Z-Ala-Ala-Ala-DL-Ala-COOEt | 0.3 | 0.14 | |
| MeO-Suc-Val-Pro-DL-Phe-COOMe | | | 1.1 |

A crystal structure of one α-ketoester bound into the active site of PPE has been completed in the Meyer laboratory and a schematic drawing of the interactions observed is shown below. The Ser-195 oxygen has added to the carbonyl group of the ketoester to form a tetrahedral intermediate which is stabilized by interactions with the oxyanion hole. This structure resembles the tetrahedral intermediate involved in peptide bond hydrolysis and proves that α-ketoesters are transition-state analogs. His-57 is hydrogen bonded to the carbonyl group of the ester functional group, the peptide backbone on a section of PPE's backbone hydrogen bonds to the inhibitor to form a β-sheet, and the benzyl ester is directed toward the S' subsites. It appears likely that the potency of this class of inhibitor could be substantially improved if the structure was extended to allow for more interactions with the S' subsites.




**Key Personnel (August 1, 1989 to March 31, 1991)**

| | | |
|---|---|---|
| James C. Powers | PI | 8% |
| Chih-Min Kam | Research Scientist | 25% |
| Masaru Yamamoto | Graduate Student | 100% |
| Andrea Lauro | Graduate Student | 42% |
| Charles Houck | Graduate Student | 100% |
| Margaret Robinson | Graduate Student | 100% |
| John Kerrigan | Graduate Student | 100% |

**Publications (1985-1991)**

1. "Irreversible Inhibition of Serine Proteases by Peptide Derivatives of α-Aminoalkylphosphonate Diphenyl Esters", Oleksyszyn, J., and Powers, J. C. (1991) *Biochemistry 30*, 485-493.

2. "Reaction of Porcine Pancreatic Elastase with 7-Substituted 3-alkoxy-4-Chloroisocoumarins: Design of Potent Inhibitors Using the Crystal Structure of the Complex Formed with 4-Chloro-3-ethoxy-7-guanidinoisocoumarin", Powers, J. C., Oleksyszyn, J., Narasimham, S. L., Kam, C-M., Radhakrishnan, R., and Meyer, E. F. (1990) *Biochemistry 29*, 3108-3118.

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**Patents Pending**

"Heterocyclic Inhibitors of Serine Proteases, J. C. Powers (1989) continuation in part filed July 1988.

"Substituted Isocoumarins," J. C. Powers, C.-M. Kam, J. Oleksyszyn, J. A. Glinski, and M. A. Hernandez (1990) continuation in part filed April 1990.

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structures, investigate the mechanisms of elastase inhibition, determine the structure of inhibitor complexes, and to understand the basic underlying mechanism by which HNE inhibitors work in animals.

**HNE Inhibitors in Animal Models.** The literature on *in vivo* testing of elastase inhibitors as preventatives of elastase-induced emphysema in laboratory animals has been reviewed by the Boston U. group (Snider et al., 1986), and they have published a number of papers in this area (Stone et al., 1981; 1990a; 1990b; Snider et al., 1985; Lucey et al., 1982; 1986; 1989; 1990). A wide variety of structures including peptide chloromethyl ketone inhibitors, transition state inhibitors, and natural and recombinant protein inhibitors have been shown to be effective in animal models of emphysema. In addition, α1-PI replacement therapy is currently undergoing trials at several centers in the U. S. for the treatment of PiZZ patients who are deficient in the inhibitor. The natural inhibitor has a 12-16 hr lifetime in plasma and must be replenished by periodic injections. Thus far, it is expensive and available only in small quantities.

## C. PROGRESS REPORT

**Specific Aims.** The major specific aim of our previous application was the development of a synthetic elastase inhibitor which would be useful for the treatment of human emphysema. A variety of inhibitor structures were proposed including heterocyclic mechanism-based inhibitors and peptide transition state analogs. We proposed to test the specificity of all new inhibitors with other PMN proteases such as human leukocyte cathepsin G. A secondary goal of the research was the extension of potent inhibitor structures to other serine proteases. We suggested that inhibitor studies would lead to a better understanding of the active site structures of the serine proteases involved in connective tissue turnover, might produce clinically useful drugs for the treatment of emphysema and related diseases, would stimulate the research of medicinal chemists in pharmaceutical companies, and will provide new tools for the *in vivo* and *in vitro* study of the role of neutrophil and mast cell proteases in a variety of physiological processes.

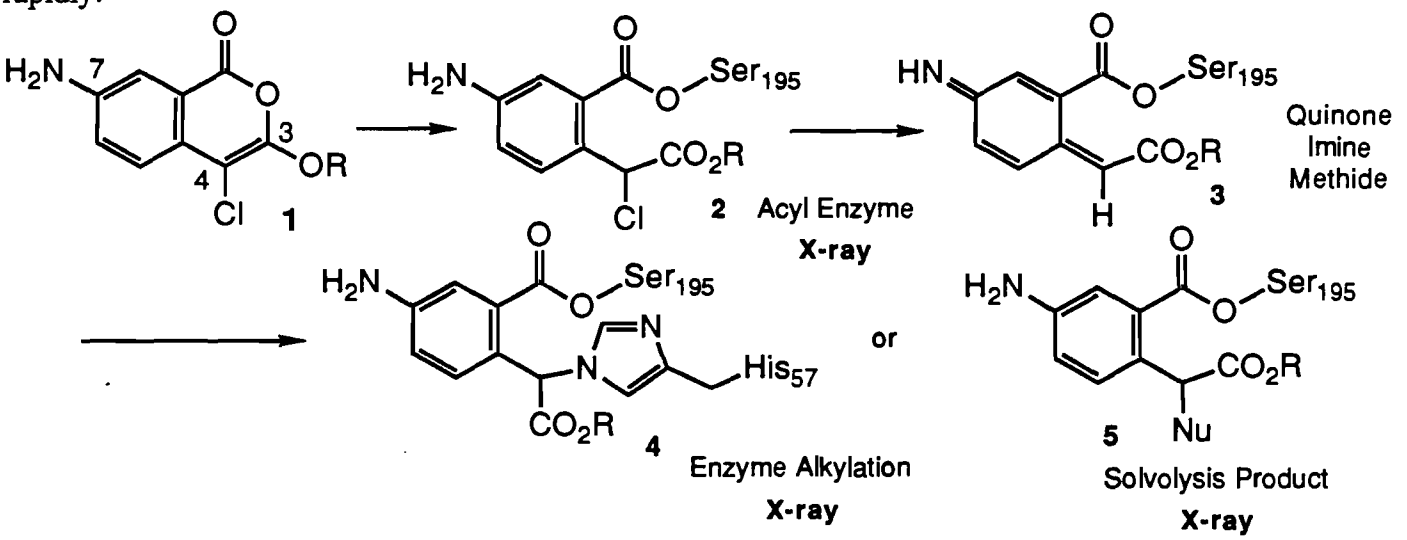
**Summary of progress.** During the 5 year period of this research project, we have developed several new classes of inhibitors for HNE including isocoumarins (mechanism-based inhibitors), peptide α-ketoesters (transition-state analogs), and peptide phosphonates (irreversible transition-state inhibitors). X-ray structural studies have been accomplished with 5 isocoumarin inhibitors bound to the active sites of PP elastase and trypsin, with 4 chloromethyl ketone inhibitors bound to the active site of HNE, and with one peptide α-ketoester inhibitor bound to the active site of PP elastase. The structural studies have led to a better understanding of the active site structures of these two important elastases and their mechanisms of inhibition. Several of the isocoumarin inhibitors developed have been studied with other physiologically important serine proteases and an influenza viral serine esterase. One of our inhibitors, 3,4-dichloroisocoumarin, is now commercially available from four companies for use as a general serine protease inhibitor. Isocoumarin and phosphonate inhibitors may prove to be valuable in other disease states related to serine protease activity and are currently being tested in animal models of sepsis and Alzheimer's disease.

The various classes of elastase inhibitors discovered in my laboratory have been used extensively by pharmaceutical companies in the development of therapeutics for the treatment of emphysema. In particular, the chloromethyl ketone inhibitor MeO-Suc-Ala-Ala-Pro-Val-$CH_2Cl$ is used as the standard elastase inhibitor for the validation of new animal models of emphysema and as a standard elastase inhibitor to compare the effectiveness of newly developed inhibitors (Fletcher et al., 1990). Benzoxazinones and peptide α-ketoesters elastase inhibitors, discovered in my laboratory, have been chosen by Syntex Canada (Krantz et al., 1987; 1990) and Merrill Dow (Peet et al., 1990) for extensive development. Peptide sequences which we discovered to be potent elastase inhibitors have been translated into the sequences found in the potent trifluoromethyl ketone inhibitors of HN elastase which are now undergoing development by ICI Americas as therapeutics (Trainor, 1987). The trifluoromethyl ketone class of elastase inhibitors is probably the closest to reaching clinical use of any of the low molecular weight inhibitors available today and at least one derivative is undergoing phase I testing.

**Isocoumarin Mechanism-Based Inhibitors.** Isocoumarins are mechanism-based (or suicide) heterocyclic serine proteases which are rich in possible masked reactive functional groups. Thus far, we have described isocoumarins which contain latent acid chloride or quinone imine methide functional groups. We have synthesized well over a hundred isocoumarin inhibitors, have published ca. 13 papers and reviews which describe these inhibitors (see publication list at end of this section) and have still have not yet reported the majority of the compounds which we have synthesized. The recent completion of several x-ray structures of

complexes of PPE inhibited by isocoumarins has yielded important insights into the binding modes of these inhibitors and we are working on two additional x-ray papers and a J. Med. Chem. paper. In addition to elastase, the isocoumarins have proved to be useful in the study of an influenza viral esterase and a group of serine proteases isolated from natural killer cells (publication 4, 8, 13, 14).

The inactivation mechanism of serine proteases by 3-alkoxy-7-amino-4-chloroisocoumarins (**1**) is shown below. The active site Ser-195 attacks the isocoumarin carbonyl group and opens the isocoumarin ring to form an acyl enzyme (**2**). This reaction unmasks a latent quinone imine methide functional group (**3**) which is formed by the elimination of HCl from the acyl enzyme. This can react either with an enzyme nucleophile (His-57) to give an irreversibly inhibited enzyme structure (**4**) or with a solvent nucleophile to give a stable acyl enzyme (**5**). Partial reactivation by hydroxylamine with some inhibited derivatives suggests a partitioning between the two enzyme-inhibitor complexes (**4** & **5**) in solution with the nonreactivatable complex (**4**) containing an alkylated histidine residue. Both the 7-amino and 4-chloro groups are required for formation of a stable inactivated enzyme; isocoumarins which lack these features inhibit serine proteases, but deacylate fairly rapidly.




In addition to the kinetic evidence, the proposed inhibition mechanism is supported by x-ray crystallographic studies. Five enzyme-isocoumarin complexes have completed in collaboration with Ed Meyer (Texas A & M) and Wolfram Bode (Max Planck Institute, Munich) and are listed below.

7-amino-4-chloro-3-methoxyisocoumarin & PPE - solvolysis product **5** with Nu = acetate - (structure published, Meyer et al., 1987).

4-chloro-3-ethoxy-7-guanidinoisocoumarin & PPE - acyl enzyme **2** with chlorine still present - structure published in 1990, publication 2.

4-chloro-3-ethoxy-7-guanidinoisocoumarin & trypsin - mixture of acyl enzyme **2** and the histidine alkylation product **4** - manuscript accepted by J. Am. Chem. Soc.

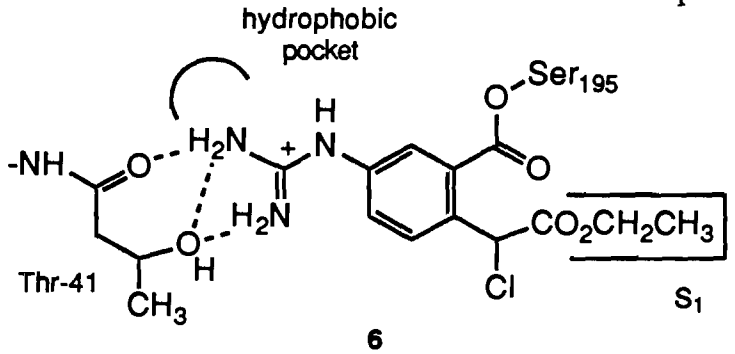
7-amino-3-bromoethoxy-4-chloroisocoumarin & PPE - histidine alkylation product **4** - structure completed and manuscript will be submitted in Aug.

7-(Tos-Phe-NH)-4-chloro-3-ethoxyisocoumarin & PPE - histidine alkylation product **4** - structure being refined.

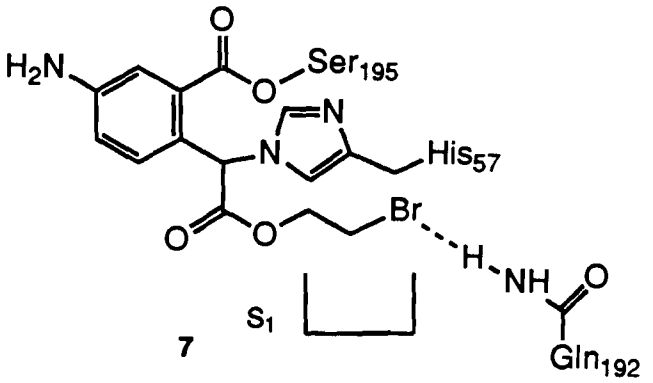
All the major products (**2**, **4**, and **5**) in the reaction scheme have been observed crystallographically with different isocoumarins. The observation of the doubly covalent bond histidine alkylation product **4** is the second time such a derivative has been observed with a serine protease. The first doubly covalently bound adduct was observed in a crystal structure of PPE inhibited by a β-lactam inhibitor which was reported by a Merck group (Navia et al., 1987). The exact product formed in the inhibition reaction varies with both the enzyme and the isocoumarin inhibitor. Different inhibitors give variable ratios of hydroxylamine reactivatable acyl enzymes (**2** or **5**) or non-reactivatable alkylation products (**4**). The solvolysis product **5** is probably an artifact of that particular crystallographic experiment which was carried out in an acetate buffer at pH 5.0, a pH where the His-57 would be protonated and less likely to undergo alkylation. Kinetic studies with a variety of inhibitors indicate that histidine alkylation is usually the favored product at neutral pH with both elastases and most of the isocoumarins studied.

**Isocoumarin Binding Modes.** The binding modes of all five isocoumarins complexed to PPE and trypsin are remarkably different even though all are tethered to Ser-195 via an ester bond. The carbonyl group of the ester bond linking Ser-195 to the inhibitor is in the oxyanion hole of the serine protease in the 7-amino-4-chloro-3-methoxyisocoumarin complex with PPE, while it is twisted out of the oxyanion hole in the 7-guanidino structure. The twisting of the ester carbonyl group allows favorable hydrogen bonding between the 7-guanidino group and Thr-41 (**6**) and explains the stability of the complex toward deacylation. His-57 is either hydrogen bonded to the ester bond or is covalently linked with the inhibitor (**4**).

Schematic drawings of the interactions observed in the PPE complexes with 4-chloro-3-ethoxy-7-guanidinoisocoumarin (**6**) and the 3-bromoethoxyisocoumarin (**7**) are shown below and a stereo drawing of 3 heterocyclic inhibitors superimposed in the active site of PPE is shown in Fig. 11 of publication 9. In the four PPE complexes, the 3-alkoxy group of the isocoumarin is either in the $S_1$ pocket or lying nearby. However in the complex of trypsin with 4-chloro-3-ethoxy-7-guanidinoisocoumarin, the 7-guanidino group of the isocoumarin is inserted into the $S_1$ pocket of trypsin to interact with Asp-189 in trypsin's binding pocket, while the 3-alkoxy group points toward the S' subsites of the enzyme. This orientation is almost 180° from the orientation found in the complex of the same compound with PPE (**6**). Thus a significantly different binding geometry has been observed for the first time when the same inhibitor is examined with different two serine proteases. It is clear that we don't know all the rules for predicting the preferred binding conformation of a small molecule inhibitor to the active site of a serine protease.




**6**




**7**

**Molecular Modeling and Inhibitor Design.** Although it is not yet possible to predict the binding mode of a new isocoumarin inhibitor to elastase, molecular modeling with x-ray crystal structures is extremely useful for improving that inhibitor and for interpreting inhibition kinetic data obtained with related inhibitor structures. For example, molecular modeling of the 7-guanidinoisocoumarin PPE complex **6** suggested that the addition of a small alkyl group (*t*-butyl) to the guanidino group might increase affinity to the enzyme due to the presence of a small hydrophobic pocket near the terminal nitrogen of guanidino group and above Thr-41. Therefore, we synthesized a series of 7-alkyl-NH-CO-NH- derivatives of 4-chloro-3-ethoxyisocoumarin. These ureas were chosen for synthesis due to the difficulty of synthesizing alkyl guanidino derivatives. Replacement of the 7-guanidino group by a urea functional group resulted in almost no loss of inhibitory potency (see following table). As predicted, the *t*-butyl-NH-CO-NH- derivative was the most effective PPE inhibitor and had a second order inhibition rate $k_{obsd}/[I]$ which was 3.5 fold higher than the parent inhibitor. Indeed, the compounds developed from the modeling are among the best irreversible inhibitors reported thus far for PPE (work is described in publication 2). In order to confirm the binding mode predicted from molecular modeling, the Meyer group is currently determining the structure of PPE inhibited by 7-(*t*-Bu-HN-CO-NH)-EtOIC.

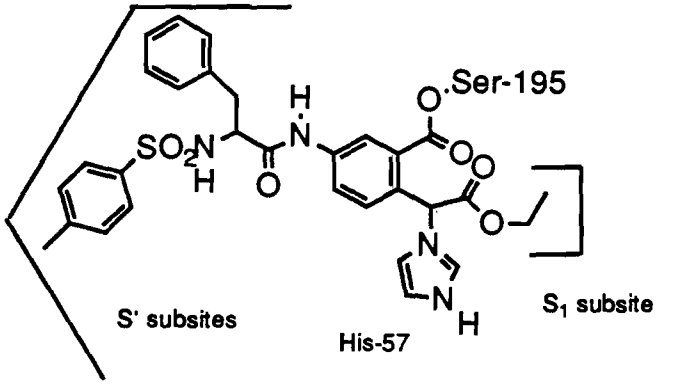
| | | |
|---|---|---|
| 7-($H_2N$-C(=$NH_2^+$)-NH)-EtOIC | 2300 $M^{-1}s^{-1}$ | |
| 7-($H_2N$-CO-NH)-EtOIC | 2200 | |
| 7-(Me-HN-CO-NH)-EtOIC | 1400 | |
| 7-(Et-HN-CO-NH)-EtOIC | 1700 | EtOIC = |
| 7-(*i*-Pr-HN-CO-NH)-EtOIC | 4900 | 4-chloro- |
| 7-(*t*-Bu-HN-CO-NH)-EtOIC | **8100** | 3-ethoxy |
| 7-(Ph-HN-CO-NH)-EtOIC | 4200 | isocoumarin |

One we realized that the 3-alkoxy group of the isocoumarin was fitting into the $S_1$ pocket of elastase, we synthesized a number of derivatives with 3-alkoxy groups of varying length. With HNE, we found the following inhibition constants: MeO, 10,000; EtO, 9,400; PrO, 54,000; and bromoethoxy, 200,000. Clearly HNE prefers the long alkoxy groups. The structure determination of the complex of the 3-

bromoethoxyisocoumarin (7) with PPE has allowed us to interpret our kinetic data with PPE (see following table, all the bromoethoxy derivatives are ca. 100 fold better with HNE, data not shown). First it is clear that the bromopropoxy derivatives with a slightly long alkoxy group are over 100 fold poorer inhibitors of PPE and HNE. All of the derivatives in which a hydrophobic group is placed on the 7-amino group are 3-7 fold better inhibitors than the parent with the exception of the phenyl urea derivative which is much poorer. The 7-amino group of the isocoumarin points toward the S' subsites of PPE and HNE and we predict that the hydrophobic groups in the extended inhibitors are interacting with the $S_2$' subsite (Leu-143, Leu-151, Thr-41 in PPE). The phenyl urea derivative is probably so rigid, that the 7-substituent can't twist and adopt a favorable conformation in the active site.

| 7-substituted-4-chloro-3-(2-bromoethoxy)isocoumarin | |
|---|---|
| 7-$NH_2$ | 1,000 $M^{-1}s^{-1}$ |
| 7-(*t*-Bu-NH-CO-NH) | 6,600 |
| 7-(Ph-NH-CO-NH) | 36 |
| 7-(Ph-$CH_2$-NH-CO-NH) | 3,010 |
| 7-(R-($C_6H_5$)($CH_3$)CH-NH-CO-NH) | 9,900 |
| 7-(Ph-$CH_2$-CO-NH) | 4,950 |
| 7-substituted-4-chloro-3-(3-bromopropoxy)isocoumarin | |
| 7-$NH_2$ | 10 |
| 7-(Ph-$CH_2$-NH-CO-NH) | 13 |
| 7-(Ph-$CH_2$-CO-NH) | 28 |

**Human Neutrophil Elastase Inhibitors.** Unfortunately, no crystal structures of an isocoumarin bound to HNE is available or likely to become available. Wolfram Bode and the Merck group have tried extensively to crystallize complexes of HNE bound to small molecule inhibitors. Up to the present, the smallest inhibitor which has yielded crystals with HNE is a tetrapeptide such as MeO-Suc-Ala-Ala-Pro-Val-$CH_2Cl$. However, the crystal structures obtained with PPE have been invaluable for modeling with HNE. The active site structures of PPE and HNE have many features in common (see the review, publication 9) and so we feel quite comfortable using the PPE crystal structures for modeling with HNE. For example, the complex of the 7-Tos-Phe derivative of 4-chloro-3-ethoxyisocoumarin has been determined and has the 3-alkoxy group in the $S_1$ pocket and the Tos-Phe group interacting with the S' subsites of PPE (shown below left). Representative inhibition rates with HNE are shown below (right). The derivatives shown all have a 3-propoxy group instead of the 3-ethoxy group shown in the crystal structure since we demonstrate that HNE preferred to an alkoxy group with one more methylene group. Clearly, most of the rates are so fast that the rate constants should be considered to be lower limits.



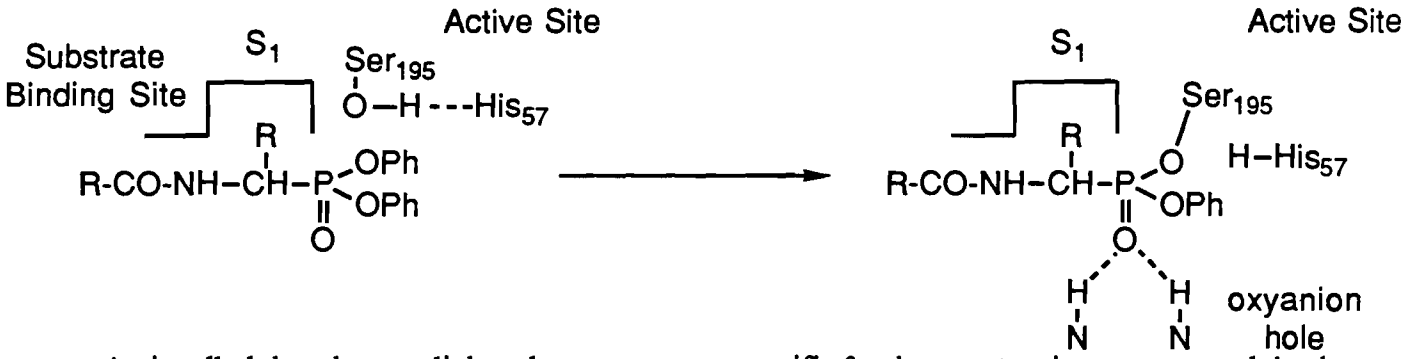

| Tos-Phe | 33,400 $M^{-1}s^{-1}$ |
|---|---|
| $CH_3CO$ | >190,000 |
| $CH_3CH_2CH_2CO$ | >124,000 |
| $(CH3)_2CHCH_2CO$ | >220,000 |
| $PhCH_2CH_2CO$ | >250,000 |
| $CH_3SCH_2CO$ | >152,000 |
| 3-$O_2N$-$C_6H_4CO$ | >210,000 |
| $CH_3CH_2OCO$ | >181,000 |
| $CH_3CH_2HNCO$ | >276,000 |
| PhNHCO | 143,000 |
| $PhCH_2NHCS$ | >131,000 |
| Ph-HNCS | >166,000 |

The active site of HNE is much more hydrophobic than that of PPE. Two significant changes are: Thr-41 in PPE is replaced in HNE by Phe-41 and Gln-192 is changed to Phe-192. Molecular modeling with HNE indicates that the Tos-Phe group is interacting with the S' subsites consisting of the side chains of Phe-41, Leu-35, and Leu-143. Clearly all the hydrophobic 7-substituents are making favorable contacts with HNE and as a result are extremely potent inhibitors.

**Dichloroisocoumarin.** We should also mention 3,4-dichloroisocoumarin which we reported as a general serine protease inhibitor in 1985 (publication 29). HLE and PPE are both inhibited effectively by 3,4-dichloroisocoumarin with $k_{obsd}/[I]$ values of 8900 and 2500 $M^{-1}s^{-1}$ respectively. Although the elastases are most effectively inhibited, dichloroisocoumarin is a general serine protease inhibitor and reacts with all serine proteases which have been investigated thus far. It is now commercially available from four companies and is becoming widely used for diagnosing new serine proteases since it is more reactive than PMSF and less hazardous than DFP. It addition it has also been invaluable to show that serine proteases are involved in

natural killer cell and cytotoxic T lymphocyte granule mediated killing of targeted cells (publications 4, 13, 14). I expect to see this inhibitor increasingly used by other investigators in the future.

**Peptide Phosphonate Inhibitors**. Peptidyl derivatives of α-aminoalkylphosphonate diphenyl ester are effective and specific inhibitors of serine proteases at low concentrations. These inhibitors are chemically stable, relatively easy to synthesize, and do not react with acetylcholinesterase. Previously synthesized phosphonate inhibitors of serine proteases include phosphonyl fluorides or phosphonamides which are unstable in solution and/or reactive toward acetylcholinesterase. Our phosphonate diphenyl esters form very stable derivatives with serine proteases due to the resemblance between the inhibition product (phosphonyl derivative shown below) and the tetrahedral intermediate involved in peptide bond hydrolysis. Our phosphonates are stable for at least 3 days in human plasma and the inhibited enzyme has a half-life for reactivation of 10 hrs in the case of chymotrypsin and >3 days in the case of the elastases. Although phosphonates are not as reactive as isocoumarins, we believe that they have considerable utility as therapeutic agents due their high stability and specificity.




Aminoalkylphosphonate diphenyl esters are very specific for the target serine protease and the data suggests that good interactions with the $S_1$ pocket of the target serine protease are necessary before nucleophilic substitution on phosphorus atom occurs to give a stable phosphonyl derivative. For example, Suc-Val-Pro-Phe$^P$(OPh)$_2$ reacts with chymotrypsin ($k_{obsd}/[I] = 44{,}000\ M^{-1}s^{-1}$) and does not react with elastases. Boc-Val-Pro-Val$^P$(OPh)$_2$ reacts with HNE ($27{,}000\ M^{-1}s^{-1}$) and PPE ($11{,}000\ M^{-1}s^{-1}$) and does not react with chymotrypsin. The longer peptides with a C-terminal phosphonate related to phenylalanine are also inhibitors for other chymotrypsin-like enzymes such as rat mast cell protease II and human cathepsin G.

| | ChyT | PPE | HNE |
|---|---|---|---|
| MeO-Suc-Ala-Ala-Pro-Nva$^P$(OPh)$_2$ | 50 | 4200 | 380 |
| MeO-Suc-Ala-Ala-Ala-Val$^P$(OPh)$_2$ | 15 | 2800 | 1500 |
| MeO-Suc-Ala-Ala-Ala-Phe$^P$(OPh)$_2$ | 2,000 | NI | NI |
| MeO-Suc-Ala-Ala-Pro-Val$^P$(OPh)$_2$ | 21 | 7,100 | 7,100 |
| MeO-Suc-Ala-Ala-Pro-Leu$^P$(OPh)$_2$ | 1,500 | 740 | 140 |
| MeO-Suc-Ala-Ala-Pro-Phe$^P$(OPh)$_2$ | 11,000 | NI | NI |
| MeO-Suc-Ala-Ala-Pro-Met$^P$(OPh)$_2$ | 570 | 44 | 53 |
| MeO-Suc-Ala-Ala-Pro-Met(O)$^P$(OPh)$_2$ | 15 | 1.6 | 1.6 |
| Boc-Val-Pro-Val$^P$(OPh)$_2$ | NI | 11,000 | 27,000 |
| Z-Phe-Pro-Phe$^P$(OPh)$_2$ | 17,000 | NI | NI |
| Suc-Val-Pro-Phe$^P$(OPh)$_2$ | 44,000 | NI | NI |

The inhibition reaction is stereospecific since $^{31}$P NMR studies have shown than only one of the two stereoisomers of Suc-Val-Pro-Phe$^P$(OPh)$_2$ reacts with chymotrypsin with a rate constant which is higher than ($146{,}000\ M^{-1}s^{-1}$) the mixture. The $^{31}$P NMR of chymotrypsin inhibited by this peptide shows one broad signal at 25.98 ppm corresponding to the Ser-195 phosphonate ester which is consistent with the product structure shown above.

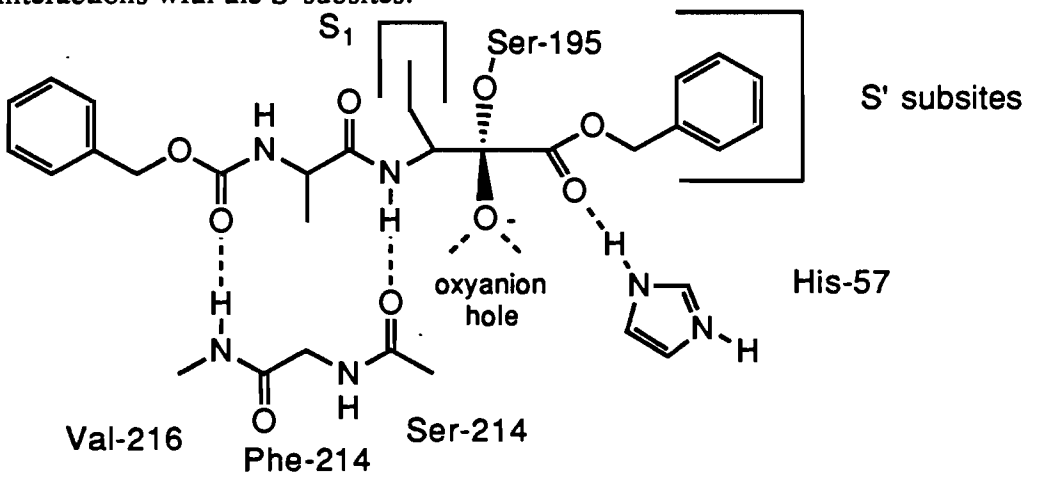
**Peptide α-Ketoester Transition-State Inhibitors.** A variety of transition-state inhibitors for elastase are currently available including peptide aldehydes, peptide boronic acids, and peptide trifluoromethyl ketones. During the course of this research, we developed novel peptide α-ketoesters as another type of transition-state inhibitor for elastases. This class of inhibitors was based on the refined x-ray crystal structure

of the complex formed between bovine trypsin and the ketoacid inhibitor 4-amidinophenylpyruvate (APPA), a potent trypsin inhibitor (Walter and Bode, 1983). In the x-ray structure, the amidinophenyl group is located in the primary specificity pocket of trypsin and the active site serine has added to the 2-carbonyl group in APPA. The oxyanion is stabilized by hydrogen bonding with groups in the oxyanion hole of trypsin. A unique feature of this structure is the hydrogen bonding observed between the carboxylate oxygen and the serine oxygen and the NH of histidine-57. We expected that the negative charge on the carboxylate would significantly contribute to the binding energy of α-ketoacids.

We designed and synthesized a number of peptide derivatives of various α-ketoacids and α-ketoesters derived from several different amino acids. Some of our kinetic results are shown in the following table where $K_I$ values are report in μM in 0.1 M Hepes, pH 7.5, 0.5 M NaCl, 9.0-9.8% $Me_2SO$ at 25°C (Ala-$CO_2Et$ = -$NHCH(CH_3)CO$-$CO_2Et$; Abu = 2-aminobutanoic acid). Surprisingly, the α-ketoesters are much better inhibitors than the corresponding acids which indicates that additional interactions with the $S_1'$ subsite and hydrogen bonding of the ester oxygen with the histidine N-H can result in significant binding energy in the case of elastase. The best inhibitors, Z-Ala-Ala-DL-Abu-COOEt, the corresponding benzyl ester, and Z-Ala-Ala-Ala-DL-Ala-COOEt, had $K_I$ values in the submicromolar range (publication 28). Importantly, MeO-Suc-Val-Pro-Phe-COOMe is one of the most potent reversible cathepsin G inhibitors known.

| Inhibitor | HLE | PPE | Cat G |
|---|---|---|---|
| Bz-DL-Ala-COOEt | 640 μM | 590 | |
| Bz-DL-Ala-COOH | 3100 | 3200 | |
| Z-Ala-Ala-DL-Abu-COOEt | 0.12 | 0.15 | |
| Z-Ala-Ala-DL-Abu-COOBzl | 0.09 | 0.08 | |
| Z-Ala-Ala-Ala-DL-Ala-COOEt | 0.3 | 0.14 | |
| MeO-Suc-Val-Pro-DL-Phe-COOMe | | | 1.1 |

A crystal structure of one α-ketoester bound into the active site of PPE has been completed in the Meyer laboratory and a schematic drawing of the interactions observed is shown below. The Ser-195 oxygen has added to the carbonyl group of the ketoester to form a tetrahedral intermediate which is stabilized by interactions with the oxyanion hole. This structure resembles the tetrahedral intermediate involved in peptide bond hydrolysis and proves that α-ketoesters are transition-state analogs. His-57 is hydrogen bonded to the carbonyl group of the ester functional group, the peptide backbone on a section of PPE's backbone hydrogen bonds to the inhibitor to form a β-sheet, and the benzyl ester is directed toward the S' subsites. It appears likely that the potency of this class of inhibitor could be substantially improved if the structure was extended to allow for more interactions with the S' subsites.




**Animal Studies With Elastase Inhibitors.** The Boston University group has tested 13 inhibitors in the hamster model and have published the results on 6 inhibitors. They propose that the *in vivo* effectiveness of a new elastase inhibitor can be predicted from *in vitro* inhibitor data and its clearance rate from the lungs. The *in vitro* assay uses $^3$H-elastin as a substrate in a physiologic buffer containing protein (bovine serum albumin). Incubation of the inhibitor with HNE in the presence of the substrate takes place for 4 hr at 37° C, a period of time thought to be similar to that required for HNE to act in the hamsters lungs (Lucey et al., 1989).

The Boston U. group classify the inhibitors into four categories. The first category includes inhibitors that are sparingly soluble in saline (approximately 1 mM or less) and are ineffective *in vivo*. For example, more than a 30:1 molar ratio of the poorly water soluble elastase inhibitor 3-chloroisocoumarin was required to achieve 50% inhibition of HNE in the *in vitro* elastin assay system. They predict that an amount of this inhibitor exceeding its solubility (1 mM in saline) would be required to obtain a 50% inhibition of instilled HNE in the hamster model, while allowing for clearance of much of the inhibitor during the 1 hr interval between instillation of inhibitor and HNE. Indeed they were not surprised that this compound was ineffective in preventing HNE-induced emphysema in the hamster (unpublished data). Possible reasons for the ineffectiveness of this category of inhibitors include their poor inhibition of HNE in saline solutions and/or the rapid clearance that is known to be associated with lipophilic compounds (Schanker, 1978).

The second category of elastase inhibitors includes irreversible inhibitors such as Suc-Ala-Ala-Pro-Val-$CH_2Cl$ (a peptide chloromethyl ketone, Lucey et al., 1989) and α1-PI (Stone et al., 1990b). The third category comprises tight binding but slowly reversible inhibitors that clear relatively slowly from the lungs. Human secretory leukocyte protease inhibitor (SLPI) and eglin-c (Snider et al., 1985; Lucey et al., 1990a) clear slowly, at least in part due to their molecular size; however, this category also includes low molecular weight inhibitors that clear slowly because of their hydrophilicity (Kennedy et al., 1987). Members of both the second and third categories show a dose-response relation in ameliorating emphysema in the hamster; compounds in the second category also demonstrate a correlation between *in vitro* inhibitory activity against HNE-induced elastin solubilization and the *in vivo* amelioration of emphysema.

Results with MeO-Suc-Ala-Ala-Pro-D,L-boro-Val pinacol ester (BOROVAL, Stone et al., 1990a) suggest a fourth category of inhibitors. BOROVAL is a highly effective but reversible transition state inhibitor of both PPE and HNE, and has been shown to prevent PPE-induced emphysema in hamsters. But pretreatment with as much as a 170-fold molar excess of BOROVAL, given intratracheally 1 h before 0.3 mg HNE, did not prevent emphysema. Indeed, lung volumes were larger after the BOROVAL pretreatment than after HNE alone. Emphysema was also induced by instilling HNE that had been mixed with and inactivated by a 41-fold molar excess of BOROVAL. Two hr after instillation of 0.3 mg HNE inactivated with a 34-fold molar excess of BOROVAL, bronchoalveolar lavage contained elastolytic activity but no evidence of hemorrhage. In contrast hemorrhage was severe in hamsters that had been instilled with 0.3 mg HNE alone.

From these studies, it was concluded that BOROVAL can enhance HNE-induced emphysema. BOROVAL is soluble in saline, highly reversible ($k_{off}$ = 0.00038 $sec^{-1}$) and rapidly cleared ($t_{1/2}$ = 15 min in the lavageable compartment of the lungs). The Boston U. group postulate that BOROVAL suppresses HNE-induced hemorrhage and the resultant influx of plasma protease inhibitors; the HNE-BOROVAL complex is transported into the alveolar interstitium, followed by dissociation of the inhibitor from the active site of HNE. Due to its small size, free BOROVAL is rapidly cleared and the reactivated HNE attacks elastic fibers giving rise to emphysema. If HNE plays a major role in the pathogenesis of emphysema and it enters the interstitium after release by neutrophils in the airways, then the administration of a reversible, rapidly cleared inhibitor to patents could exacerbate the emphysema.

**Acute Lung Injury Induced by Pulmonary Neutrophilia.** In order to better understand the interactions among the alveolar wall, pulmonary neutrophil, enzyme release, acute lung injury and airspace enlargement, the Boston U. group developed a model of pulmonary neutrophilia induced by intratracheal instillation of E. coli lipopolysaccharide (LPS) in hamsters. The instillation of 10-100 ug of LPS induces pulmonary neutrophilia, which peaks at about 24 hrs; 75-100 x $10^6$ neutrophils (PMN) are recoverable by bronchoalveolar lavage (BAL). There is also evidence of acute lung injury as indicated by the presence of blood and increased proteins in the BAL fluid; elastolytic activity measured with tritiated elastin as substrate is not present in BAL. The intratracheal instillation of CHO-Met-Leu-Phe at 24 hrs does not give rise to further increase in BAL PMN counts, but results in measurable elastolytic activity and an increase in blood and protein in the BAL fluid. Beta-glucuronidase, an index of PMN degranulation, is increased in the BAL fluid after CHO-Met-Leu-Phe treatment. There is an approximately 20% increase in mean linear intercept at 28-32 hrs after administration of LPS; the airspace enlargement is not enhanced by administration of CHO-Met-Leu-Phe at 24 hrs.

Instillation of recombinant human α1-PI or SLPI with the CHO-Met-Leu-Phe prevents the increase in blood and protein in the BAL fluid; that neutrophil degranulation is not affected is indicated by absence of change in the level of the BAL beta-glucuronidase. We proposed to use this model to test the efficacy of

elastase inhibitors in protecting against the induction of acute lung injury by degranulation of the animals' own pulmonary neutrophils. It may also prove useful as a model for testing these agents for their effects in protecting against PMN-induced respiratory airspace enlargement.

**Key Personnel (August 1, 1989 to July 31, 1990)**

| | | |
|---|---|---|
| James C. Powers | PI | 8% |
| Chih-Min Kam | Research Scientist | 25% |
| Masaru Yamamoto | Graduate Student | 100% |
| Andrea Lauro | Graduate Student | 42% |
| Charles Houck | Graduate Student | 100% |
| Margaret Robinson | Graduate Student | 100% |

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"Thioester Inhibitors of Serine Proteases", J. C. Powers (1986) U.S. Patent 4,585,793.

"Heterocyclic Inhibitors of Serine Proteases", J. C. Powers and J. W. Harper (1986) U.S. Patent 4,596,822.

"Aryl Sulfonyl Fluoride Compounds", J. C. Powers (1987) U. S. Patent 4,659,855.

"Aryl Sulfonyl Fluoride Inhibitors of Elastase and Chymotrypsin-like Enzymes", J. C. Powers (1988) U. S. Patent 4,725,545.

"Heterocyclic Inhibitors of Serine Proteases, J. C. Powers (1989) U. S. Patent 4,847,202.

**Patents Pending**

"Heterocyclic Inhibitors of Serine Proteases, J. C. Powers (1989) continuation in part filed July 1988.

"Substituted Isocoumarins," J. C. Powers, C.-M. Kam, J. Oleksyszyn, J. A. Glinski, and M. A. Hernandez (1990) continuation in part filed April 1990.

"Substituted Isocoumarins as Serine Protease Inhibitors, and Anti-inflammatory Agents," J. C. Powers, C.-M. Kam, J. Oleksyszyn, J. A. Glinski, and M. A. Hernandez (1990) continuation in part filed April 1990.

## D. EXPERIMENTAL DESIGN AND METHODS.

The proposed research represents an integrated proposal from three quite different research groups. The PI's laboratory will carry out the design and synthesis of all new inhibitors structures, study their inhibition mechanism *in vitro*, and measure their inhibitory potency *in vitro*. He will also study the interaction of the oxidizable inhibitors with myeloperoxidase. The Meyer group will carry out x-ray crystallographic studies with the elastase inhibitors using HNE and PPE, and will model new inhibitor structures (along with the PI) into the active site of both PPE and HLE. The Boston U. group will assay appropriate elastase inhibitors *in vivo* in the elastin assay and will perform all the proposed animal studies. The results from the animal studies will be feedback into the design and synthesis activities to produce improved generations of inhibitors for further animal studies.

### 1. Develop and Synthesize Potent Synthetic Elastase Inhibitors Which Lose Their Inhibitory Potency Upon Oxidation.

Most of the low-molecular weight inhibitors which have been developed by the academic and industrial investigators are stable toward oxidation by biological systems. And yet, the natural inhibitor of HNE, α1-PI is inactivated by oxidation due to the presence of Met-358 in active site of α1-PI. We believe that this susceptibility to oxidation is essential for the neutrophil's ability to migrate to sites of infection and attack invading microbes. We propose to develop low molecular wt. inhibitors of HNE which lose their elastase inhibitory ability upon oxidation. Once the design principles for this type of inhibitor are developed, we feel that pharmaceutical companies will add such structural features to the drugs currently under development.

# Synthetic Elastase Inhibitors (HL29307)
# Final Report (August 15, 1989-July 31, 1991)

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**Specific Aims.** The major specific aim of our proposed research was the development of a synthetic elastase inhibitor which would be useful for the treatment of human emphysema. A variety of inhibitor structures were proposed including heterocyclic mechanism-based inhibitors and peptide transition state analogs. We proposed to test the specificity of all new inhibitors with other PMN proteases such as human leukocyte cathepsin G. A secondary goal of the research was the extension of potent inhibitor structures to other serine proteases. We suggested that inhibitor studies would lead to a better understanding of the active site structures of the serine proteases involved in connective tissue turnover, might produce clinically useful drugs for the treatment of emphysema and related diseases, would stimulate the research of medicinal chemists in pharmaceutical companies, and will provide new tools for the *in vivo* and *in vitro* study of the role of neutrophil and mast cell proteases in a variety of physiological processes.

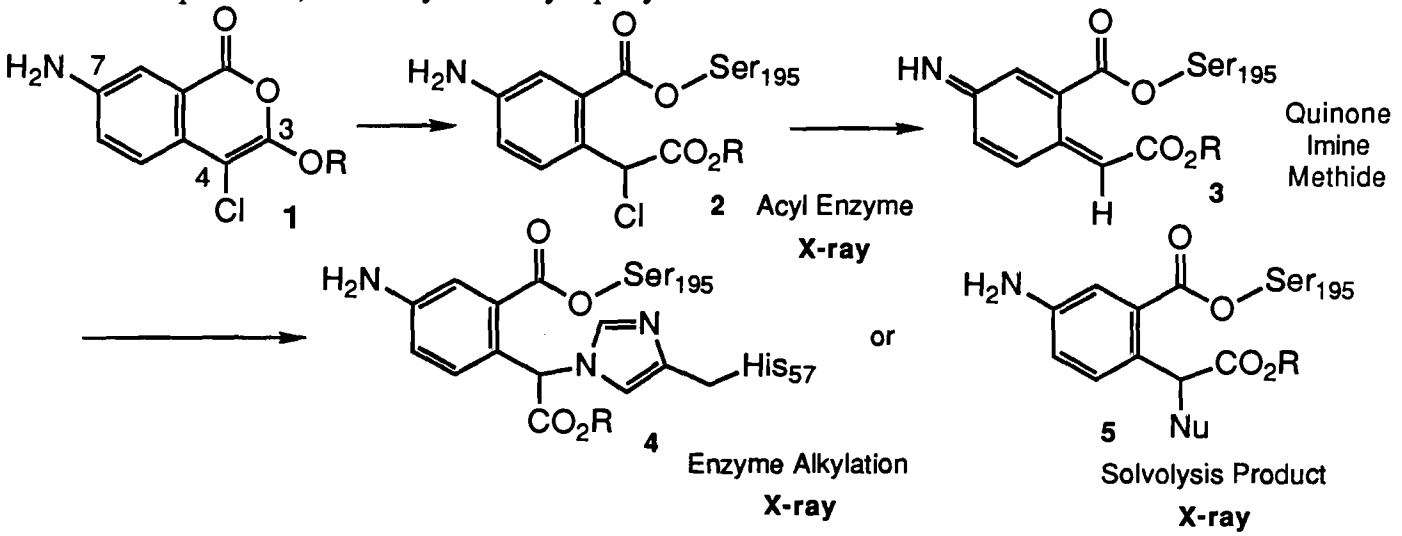
**Summary of progress.** During the 5 year period of this research project, we have developed several new classes of inhibitors for HNE including isocoumarins (mechanism-based inhibitors), peptide α-ketoesters (transition-state analogs), and peptide phosphonates (irreversible transition-state inhibitors). X-ray structural studies have been accomplished with 5 isocoumarin inhibitors bound to the active sites of PP elastase and trypsin, with 4 chloromethyl ketone inhibitors bound to the active site of HNE, and with one peptide α-ketoester inhibitor bound to the active site of PP elastase. The structural studies have led to a better understanding of the active site structures of these two important elastases and their mechanisms of inhibition. Several of the isocoumarin inhibitors developed have been studied with other physiologically important serine proteases and an influenza viral serine esterase. One of our inhibitors, 3,4-dichloroisocoumarin, is now commercially available from four companies for use as a general serine protease inhibitor. Isocoumarin and phosphonate inhibitors may prove to be valuable in other disease states related to serine protease activity and are currently being tested in animal models of sepsis and Alzheimer's disease.

The various classes of elastase inhibitors discovered in my laboratory have been used extensively by pharmaceutical companies in the development of therapeutics for the treatment of emphysema. In particular, the chloromethyl ketone inhibitor MeO-Suc-Ala-Ala-Pro-Val-$CH_2Cl$ is used as the standard elastase inhibitor for the validation of new animal models of emphysema and as a standard elastase inhibitor to compare the effectiveness of newly developed inhibitors (Fletcher et al., 1990). Benzoxazinones and peptide α-ketoesters elastase inhibitors, discovered in my laboratory, have been chosen by Syntex Canada (Krantz et al., 1987; 1990) and Merrill Dow (Peet et al., 1990) for extensive development. Peptide sequences which we discovered to be potent elastase inhibitors have been translated into the sequences found in the potent trifluoromethyl ketone inhibitors of HN elastase which are now undergoing development by ICI Americas as therapeutics (Trainor, 1987). The trifluoromethyl ketone class of elastase inhibitors is probably the closest to reaching clinical use of any of the low molecular weight inhibitors available today and at least one derivative is undergoing phase I testing.

**Isocoumarin Mechanism-Based Inhibitors.** Isocoumarins are mechanism-based (or suicide) heterocyclic serine proteases which are rich in possible masked reactive functional groups. Thus far, we have described isocoumarins which contain latent acid chloride or quinone imine methide functional groups. We have synthesized well over a hundred isocoumarin inhibitors, have published ca. 13 papers and reviews which describe these inhibitors (see publication list at end of this section) and have still have not yet reported the majority of the compounds which we have synthesized. The recent completion of several x-ray structures of complexes of PPE inhibited by isocoumarins has yielded important insights into the binding modes of these inhibitors and we are working on two additional x-ray papers and a J. Med. Chem. paper. In addition to elastase, the isocoumarins have proved to be useful in

the study of an influenza viral esterase and a group of serine proteases isolated from natural killer cells (publication 4, 8, 13, 14).

The inactivation mechanism of serine proteases by 3-alkoxy-7-amino-4-chloroisocoumarins (**1**) is shown below. The active site Ser-195 attacks the isocoumarin carbonyl group and opens the isocoumarin ring to form an acyl enzyme (**2**). This reaction unmasks a latent quinone imine methide functional group (**3**) which is formed by the elimination of HCl from the acyl enzyme. This can react either with an enzyme nucleophile (His-57) to give an irreversibly inhibited enzyme structure (**4**) or with a solvent nucleophile to give a stable acyl enzyme (**5**). Partial reactivation by hydroxylamine with some inhibited derivatives suggests a partitioning between the two enzyme-inhibitor complexes (**4** & **5**) in solution with the nonreactivatable complex (**4**) containing an alkylated histidine residue. Both the 7-amino and 4-chloro groups are required for formation of a stable inactivated enzyme; isocoumarins which lack these features inhibit serine proteases, but deacylate fairly rapidly.




In addition to the kinetic evidence, the proposed inhibition mechanism is supported by x-ray crystallographic studies. Five enzyme-isocoumarin complexes have completed in collaboration with Ed Meyer (Texas A & M) and Wolfram Bode (Max Planck Institute, Munich) and are listed below.

7-amino-4-chloro-3-methoxyisocoumarin & PPE - solvolysis product **5** with Nu = acetate - (structure published, Meyer et al., 1987).

4-chloro-3-ethoxy-7-guanidinoisocoumarin & PPE - acyl enzyme **2** with chlorine still present - structure published in 1990, publication 2.

4-chloro-3-ethoxy-7-guanidinoisocoumarin & trypsin - mixture of acyl enzyme **2** and the histidine alkylation product **4** - structure published in J. Am. Chem. Soc.

7-amino-3-bromoethoxy-4-chloroisocoumarin & PPE - histidine alkylation product **4** - structure published.

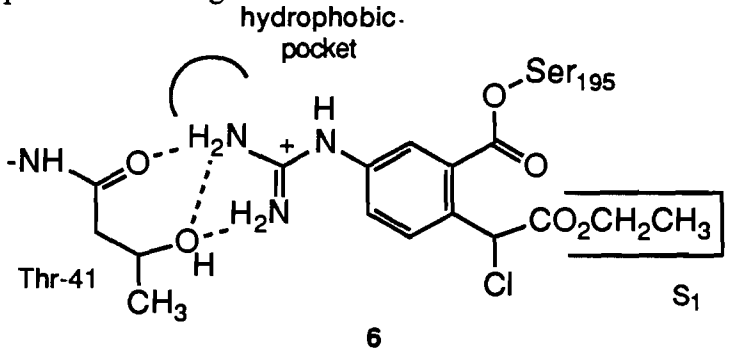
7-(Tos-Phe-NH)-4-chloro-3-ethoxyisocoumarin & PPE - histidine alkylation product **4** - structure complete and will be submitted soon.

All the major products (**2**, **4**, and **5**) in the reaction scheme have been observed crystallographically with different isocoumarins. The observation of the doubly covalent bond histidine alkylation product **4** is the second time such a derivative has been observed with a serine protease. The first doubly covalently bound adduct was observed in a crystal structure of PPE inhibited by a β-lactam inhibitor which was reported by a Merck group (Navia et al., 1987). The exact product formed in the inhibition reaction varies with both the enzyme and the isocoumarin inhibitor. Different inhibitors give variable ratios of hydroxylamine reactivatable acyl enzymes (**2** or **5**) or non-reactivatable alkylation products (**4**). The solvolysis product **5** is probably an artifact of that particular crystallographic experiment which was carried out in an acetate buffer at pH 5.0, a pH where the His-57 would be

protonated and less likely to undergo alkylation. Kinetic studies with a variety of inhibitors indicate that histidine alkylation is usually the favored product at neutral pH with both elastases and most of the isocoumarins studied.

**Isocoumarin Binding Modes.** The binding modes of all five isocoumarins complexed to PPE and trypsin are remarkably different even though all are tethered to Ser-195 via an ester bond. The carbonyl group of the ester bond linking Ser-195 to the inhibitor is in the oxyanion hole of the serine protease in the 7-amino-4-chloro-3-methoxyisocoumarin complex with PPE, while it is twisted out of the oxyanion hole in the 7-guanidino structure. The twisting of the ester carbonyl group allows favorable hydrogen bonding between the 7-guanidino group and Thr-41 (**6**) and explains the stability of the complex toward deacylation. His-57 is either hydrogen bonded to the ester bond or is covalently linked with the inhibitor (**4**).

Schematic drawings of the interactions observed in the PPE complexes with 4-chloro-3-ethoxy-7-guanidinoisocoumarin (**6**) and the 3-bromoethoxyisocoumarin (**7**) are shown below and a stereo drawing of 3 heterocyclic inhibitors superimposed in the active site of PPE is shown in Fig. 11 of publication 9. In the four PPE complexes, the 3-alkoxy group of the isocoumarin is either in the $S_1$ pocket or lying nearby. However in the complex of trypsin with 4-chloro-3-ethoxy-7-guanidinoisocoumarin, the 7-guanidino group of the isocoumarin is inserted into the $S_1$ pocket of trypsin to interact with Asp-189 in trypsin's binding pocket, while the 3-alkoxy group points toward the S' subsites of the enzyme. This orientation is almost 180° from the orientation found in the complex of the same compound with PPE (**6**). Thus a significantly different binding geometry has been observed for the first time when the same inhibitor is examined with different two serine proteases. It is clear that we don't know all the rules for predicting the preferred binding conformation of a small molecule inhibitor to the active site of a serine protease.

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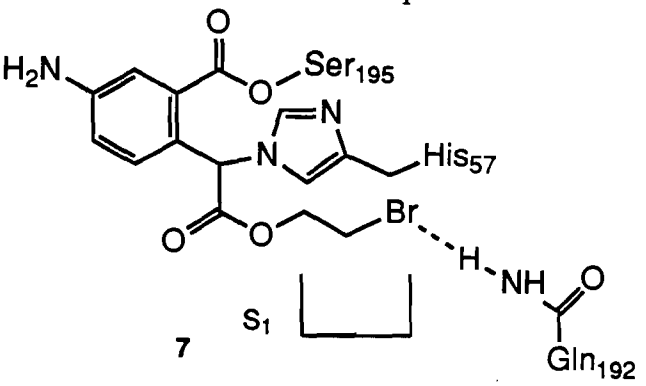


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**Molecular Modeling and Inhibitor Design.** Although it is not yet possible to predict the binding mode of a new isocoumarin inhibitor to elastase, molecular modeling with x-ray crystal structures is extremely useful for improving that inhibitor and for interpreting inhibition kinetic data obtained with related inhibitor structures. For example, molecular modeling of the 7-guanidinoisocoumarin PPE complex **6** suggested that the addition of a small alkyl group (*t*-butyl) to the guanidino group might increase affinity to the enzyme due to the presence of a small hydrophobic pocket near the terminal nitrogen of guanidino group and above Thr-41. Therefore, we synthesized a series of 7-alkyl-NH-CO-NH- derivatives of 4-chloro-3-ethoxyisocoumarin. These ureas were chosen for synthesis due to the difficulty of synthesizing alkyl guanidino derivatives. Replacement of the 7-guanidino group by a urea functional group resulted in almost no loss of inhibitory potency (see following table). As predicted, the *t*-butyl-NH-CO-NH- derivative was the most effective PPE inhibitor and had a second order inhibition rate $k_{obsd}/[I]$ which was 3.5 fold higher than the parent inhibitor. Indeed, the compounds developed from the modeling are among the best irreversible inhibitors reported thus far for PPE (work is described in publication 2). In order to confirm the binding mode predicted from molecular modeling, the Meyer group is currently determining the structure of PPE inhibited by 7-(*t*-Bu-HN-CO-NH)-EtOIC.

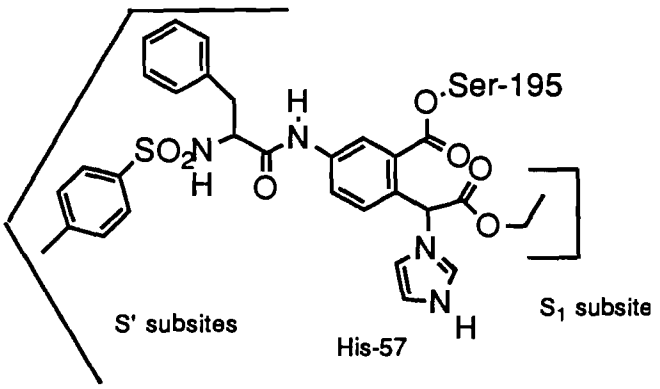
| | | |
|---|---|---|
| 7-($H_2N$-C(=$NH_2^+$)-NH)-EtOIC | 2300 $M^{-1}s^{-1}$ | |
| 7-($H_2N$-CO-NH)-EtOIC | 2200 | |
| 7-(Me-HN-CO-NH)-EtOIC | 1400 | |
| 7-(Et-HN-CO-NH)-EtOIC | 1700 | EtOIC = |
| 7-(*i*-Pr-HN-CO-NH)-EtOIC | 4900 | 4-chloro- |
| 7-(*t*-Bu-HN-CO-NH)-EtOIC | **8100** | 3-ethoxy |
| 7-(Ph-HN-CO-NH)-EtOIC | 4200 | isocoumarin |

One we realized that the 3-alkoxy group of the isocoumarin was fitting into the $S_1$ pocket of elastase, we synthesized a number of derivatives with 3-alkoxy groups of varying length. With HNE, we found the following inhibition constants: MeO, 10,000; EtO, 9,400; PrO, 54,000; and bromoethoxy, 200,000. Clearly HNE prefers the long alkoxy groups. The structure determination of the complex of the 3-bromoethoxyisocoumarin (**7**) with PPE has allowed us to interpret our kinetic data with PPE (see following table, all the bromoethoxy derivatives are ca. 100 fold better with HNE, data not shown). First it is clear that the bromopropoxy derivatives with a slightly long alkoxy group are over 100 fold poorer inhibitors of PPE and HNE. All of the derivatives in which a hydrophobic group is placed on the 7-amino group are 3-7 fold better inhibitors than the parent with the exception of the phenyl urea derivative which is much poorer. The 7-amino group of the isocoumarin points toward the S' subsites of PPE and HNE and we predict that the hydrophobic groups in the extended inhibitors are interacting with the $S_2'$ subsite (Leu-143, Leu-151, Thr-41 in PPE). The phenyl urea derivative is probably so rigid, that the 7-substituent can't twist and adopt a favorable conformation in the active site.

| | |
|---|---|
| 7-substituted-4-chloro-3-(2-bromoethoxy)isocoumarin | |
| 7-$NH_2$ | 1,000 $M^{-1}s^{-1}$ |
| 7-(*t*-Bu-NH-CO-NH) | 6,600 |
| 7-(Ph-NH-CO-NH) | 36 |
| 7-(Ph-$CH_2$-NH-CO-NH) | 3,010 |
| 7-(R-($C_6H_5$)($CH_3$)CH-NH-CO-NH) | 9,900 |
| 7-(Ph-$CH_2$-CO-NH) | 4,950 |
| 7-substituted-4-chloro-3-(3-bromopropoxy)isocoumarin | |
| 7-$NH_2$ | 10 |
| 7-(Ph-$CH_2$-NH-CO-NH) | 13 |
| 7-(Ph-$CH_2$-CO-NH) | 28 |

**Human Neutrophil Elastase Inhibitors.** Unfortunately, no crystal structures of an isocoumarin bound to HNE is available or likely to become available. Wolfram Bode and the Merck group have tried extensively to crystallize complexes of HNE bound to small molecule inhibitors. Up to the present, the smallest inhibitor which has yielded crystals with HNE is a tetrapeptide such as MeO-Suc-Ala-Ala-Pro-Val-$CH_2Cl$. However, the crystal structures obtained with PPE have been invaluable for modeling with HNE. The active site structures of PPE and HNE have many features in common (see the review, publication 9) and so we feel quite comfortable using the PPE crystal structures for modeling with HNE. For example, the complex of the 7-Tos-Phe derivative of 4-chloro-3-ethoxyisocoumarin has been determined and has the 3-alkoxy group in the $S_1$ pocket and the Tos-Phe group interacting with the S' subsites of PPE (shown below left). Representative inhibition rates with HNE are shown below (right). The derivatives shown all have a 3-propoxy group instead of the 3-ethoxy group shown in the crystal structure since we demonstrate that HNE preferred to an alkoxy group with one more methylene group. Clearly, most of the rates are so fast that the rate constants should be considered to be lower limits.

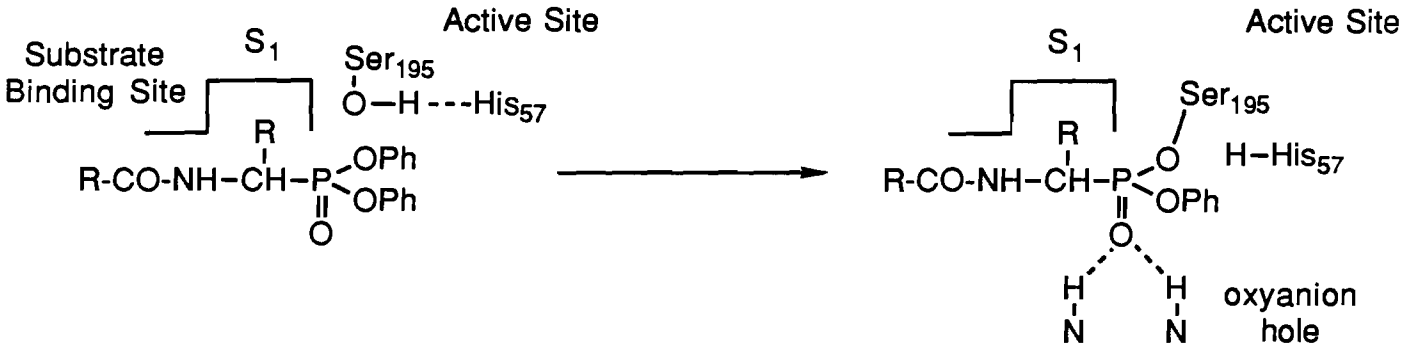
The active site of HNE is much more hydrophobic than that of PPE. Two significant changes are: Thr-41 in PPE is replaced in HNE by Phe-41 and Gln-192 is changed to Phe-192. Molecular modeling with HNE indicates that the Tos-Phe group is interacting with the S' subsites consisting of the side chains of Phe-41, Leu-35, and Leu-143. Clearly all the hydrophobic 7-substituents are making favorable contacts with HNE and as a result are extremely potent inhibitors.




| | |
|---|---|
| Tos-Phe | 33,400 $M^{-1}s^{-1}$ |
| $CH_3CO$ | >190,000 |
| $CH_3CH_2CH_2CO$ | >124,000 |
| $(CH3)_2CHCH_2CO$ | >220,000 |
| $PhCH_2CH_2CO$ | >250,000 |
| $CH_3SCH_2CO$ | >152,000 |
| $3\text{-}O_2N\text{-}C_6H_4CO$ | >210,000 |
| $CH_3CH_2OCO$ | >181,000 |
| $CH_3CH_2HNCO$ | >276,000 |
| PhNHCO | 143,000 |
| $PhCH_2NHCS$ | >131,000 |
| Ph-HNCS | >166,000 |

**Dichloroisocoumarin.** We should also mention 3,4-dichloroisocoumarin which we reported as a general serine protease inhibitor in 1985 (publication 29). HLE and PPE are both inhibited effectively by 3,4-dichloroisocoumarin with $k_{obsd}/[I]$ values of 8900 and 2500 $M^{-1}s^{-1}$ respectively. Although the elastases are most effectively inhibited, dichloroisocoumarin is a general serine protease inhibitor and reacts with all serine proteases which have been investigated thus far. It is now commercially available from four companies and is becoming widely used for diagnosing new serine proteases since it is more reactive than PMSF and less hazardous than DFP. It addition it has also been invaluable to show that serine proteases are involved in natural killer cell and cytotoxic T lymphocyte granule mediated killing of targeted cells (publications 4, 13, 14). I expect to see this inhibitor increasingly used by other investigators in the future.

**Peptide Phosphonate Inhibitors.** Peptidyl derivatives of α-aminoalkylphosphonate diphenyl ester are effective and specific inhibitors of serine proteases at low concentrations. These inhibitors are chemically stable, relatively easy to synthesize, and do not react with acetylcholinesterase. Previously synthesized phosphonate inhibitors of serine proteases include phosphonyl fluorides or phosphonamides which are unstable in solution and/or reactive toward acetylcholinesterase. Our phosphonate diphenyl esters form very stable derivatives with serine proteases due to the resemblance between the inhibition product (phosphonyl derivative shown below) and the tetrahedral intermediate involved in peptide bond hydrolysis. Our phosphonates are stable for at least 3 days in human plasma and the inhibited enzyme has a half-life for reactivation of 10 hrs in the case of chymotrypsin and >3 days in the case of the elastases. Although phosphonates are not as reactive as isocoumarins, we believe that they have considerable utility as therapeutic agents due their high stability and specificity.




Aminoalkylphosphonate diphenyl esters are very specific for the target serine protease and the data suggests that good interactions with the $S_1$ pocket of the target serine protease are necessary before nucleophilic substitution on phosphorus atom occurs to give a stable phosphonyl derivative. For example, Suc-Val-Pro-$Phe^P(OPh)_2$ reacts with chymotrypsin ($k_{obsd}/[I]$ = 44,000 $M^{-1}s^{-1}$) and does not react with elastases. Boc-Val-Pro-$Val^P(OPh)_2$ reacts with HNE (27,000 $M^{-1}s^{-1}$) and PPE (11,000 $M^{-1}s^{-1}$) and does not react with chymotrypsin. The longer peptides with a C-terminal phosphonate related to

phenylalanine are also inhibitors for other chymotrypsin-like enzymes such as rat mast cell protease II and human cathepsin G.

| | ChyT | PPE | HNE |
|---|---|---|---|
| MeO-Suc-Ala-Ala-Pro-Nva$^{P}$(OPh)$_2$ | 50 | 4200 | 380 |
| MeO-Suc-Ala-Ala-Ala-Val$^{P}$(OPh)$_2$ | 15 | 2800 | 1500 |
| MeO-Suc-Ala-Ala-Ala-Phe$^{P}$(OPh)$_2$ | 2,000 | NI | NI |
| MeO-Suc-Ala-Ala-Pro-Val$^{P}$(OPh)$_2$ | 21 | 7,100 | 7,100 |
| MeO-Suc-Ala-Ala-Pro-Leu$^{P}$(OPh)$_2$ | 1,500 | 740 | 140 |
| MeO-Suc-Ala-Ala-Pro-Phe$^{P}$(OPh)$_2$ | 11,000 | NI | NI |
| MeO-Suc-Ala-Ala-Pro-Met$^{P}$(OPh)$_2$ | 570 | 44 | 53 |
| MeO-Suc-Ala-Ala-Pro-Met(O)$^{P}$(OPh)$_2$ | 15 | 1.6 | 1.6 |
| Boc-Val-Pro-Val$^{P}$(OPh)$_2$ | NI | 11,000 | 27,000 |
| Z-Phe-Pro-Phe$^{P}$(OPh)$_2$ | 17,000 | NI | NI |
| Suc-Val-Pro-Phe$^{P}$(OPh)$_2$ | 44,000 | NI | NI |

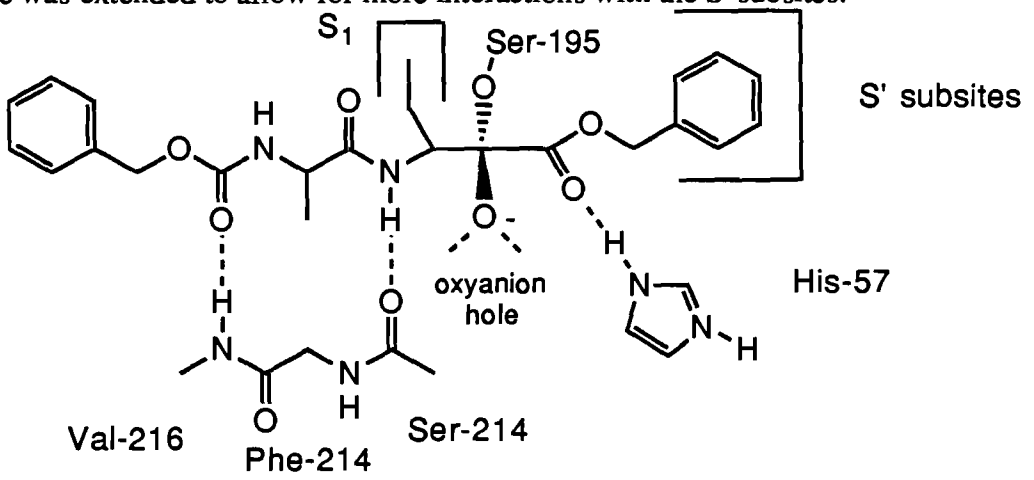
The inhibition reaction is stereospecific since $^{31}$P NMR studies have shown than only one of the two stereoisomers of Suc-Val-Pro-Phe$^{P}$(OPh)$_2$ reacts with chymotrypsin with a rate constant which is higher than (146,000 $M^{-1}s^{-1}$) the mixture. The $^{31}$P NMR of chymotrypsin inhibited by this peptide shows one broad signal at 25.98 ppm corresponding to the Ser-195 phosphonate ester which is consistent with the product structure shown above.

**Peptide α-Ketoester Transition-State Inhibitors.** A variety of transition-state inhibitors for elastase are currently available including peptide aldehydes, peptide boronic acids, and peptide trifluoromethyl ketones. During the course of this research, we developed novel peptide α-ketoesters as another type of transition-state inhibitor for elastases. This class of inhibitors was based on the refined x-ray crystal structure of the complex formed between bovine trypsin and the ketoacid inhibitor 4-amidinophenylpyruvate (APPA), a potent trypsin inhibitor (Walter and Bode, 1983). In the x-ray structure, the amidinophenyl group is located in the primary specificity pocket of trypsin and the active site serine has added to the 2-carbonyl group in APPA. The oxyanion is stabilized by hydrogen bonding with groups in the oxyanion hole of trypsin. A unique feature of this structure is the hydrogen bonding observed between the carboxylate oxygen and the serine oxygen and the NH of histidine-57. We expected that the negative charge on the carboxylate would significantly contribute to the binding energy of α-ketoacids.

We designed and synthesized a number of peptide derivatives of various α-ketoacids and α-ketoesters derived from several different amino acids. Some of our kinetic results are shown in the following table where $K_I$ values are report in μM in 0.1 M Hepes, pH 7.5, 0.5 M NaCl, 9.0-9.8% $Me_2SO$ at 25°C (Ala-$CO_2$Et = -NHCH($CH_3$)CO-$CO_2$Et; Abu = 2-aminobutanoic acid). Surprisingly, the α-ketoesters are much better inhibitors than the corresponding acids which indicates that additional interactions with the $S_1$' subsite and hydrogen bonding of the ester oxygen with the histidine N-H can result in significant binding energy in the case of elastase. The best inhibitors, Z-Ala-Ala-DL-Abu-COOEt, the corresponding benzyl ester, and Z-Ala-Ala-Ala-DL-Ala-COOEt, had $K_I$ values in the submicromolar range (publication 28). Importantly, MeO-Suc-Val-Pro-Phe-COOMe is one of the most potent reversible cathepsin G inhibitors known.

| Inhibitor | HLE | PPE | Cat G |
|---|---|---|---|
| Bz-DL-Ala-COOEt | 640 μM | 590 | |
| Bz-DL-Ala-COOH | 3100 | 3200 | |
| Z-Ala-Ala-DL-Abu-COOEt | 0.12 | 0.15 | |
| Z-Ala-Ala-DL-Abu-COOBzl | 0.09 | 0.08 | |
| Z-Ala-Ala-Ala-DL-Ala-COOEt | 0.3 | 0.14 | |
| MeO-Suc-Val-Pro-DL-Phe-COOMe | | | 1.1 |

A crystal structure of one α-ketoester bound into the active site of PPE has been completed in the Meyer laboratory and a schematic drawing of the interactions observed is shown below. The Ser-195 oxygen has added to the carbonyl group of the ketoester to form a tetrahedral intermediate which is stabilized by interactions with the oxyanion hole. This structure resembles the tetrahedral intermediate involved in peptide bond hydrolysis and proves that α-ketoesters are transition-state analogs. His-57 is hydrogen bonded to the carbonyl group of the ester functional group, the peptide backbone on a section of PPE's backbone hydrogen bonds to the inhibitor to form a β-sheet, and the benzyl ester is directed toward the S' subsites. It appears likely that the potency of this class of inhibitor could be substantially improved if the structure was extended to allow for more interactions with the S' subsites.




**Key Personnel (August 1, 1990 to July 31, 1991)**

| | | |
|---|---|---|
| James C. Powers | PI | 8% |
| Masaru Yamamoto | Graduate Student | 100% |
| Charles Houck | Graduate Student | 16% |
| John Kerrigan | Graduate Student | 100% |

**Publications (1985-1991)**


1. "Irreversible Inhibition of Serine Proteases by Peptide Derivatives of α-Aminoalkylphosphonate Diphenyl Esters", Oleksyszyn, J., and Powers, J. C. (1991) *Biochemistry 30*, 485-493.

2. "Reaction of Porcine Pancreatic Elastase with 7-Substituted 3-alkoxy-4-Chloroisocoumarins: Design of Potent Inhibitors Using the Crystal Structure of the Complex Formed with 4-Chloro-3-ethoxy-7-guanidinoisocoumarin", Powers, J. C., Oleksyszyn, J., Narasimham, S. L., Kam, C-M., Radhakrishnan, R., and Meyer, E. F. (1990) *Biochemistry 29*, 3108-3118.

3. "Physiologically Important Peptidases and Proteases: Ideal Targets for the Design of New Therapeutics", Powers, J. C., Kam, C-M., Oleksyszyn, J., and Ueda, T. (1990) *Peptides, Proceedings of the Eleventh American Peptide Symposium* (Rivier, J. E., and Marshall, G. R., Eds.), pp. 38-42, ESCOM, Leiden.

4. "Selective Isocoumarin Serine Protease Inhibitors Block RNK-16 Lymphocyte Granule-Mediated Cytolysis", Hudig, D., Allison, N. J., Kam, C-M., and Powers, J. C. (1989) *Mol. Immunology*, 26, 793-798.

5. "Amelioration of Human Neutrophil Elastase-Induced Emphysema in Hamsters by Pretreatment with an Oligopeptide Chloromethyl Ketone", Lucey, E. C., Stone, P. J., Powers, J. C., and Snider, G. L. (1989) *Eur. Respir. J.*, 2, 421-427.

6. "Irreversible Inhibition of Serine Proteases by Peptidyl Derivatives of α-Aminoalkylphosphonate Diphenyl Esters", Oleksyszyn, J., and Powers, J. C. (1989) *Biochem. Biophys. Res. Commun. 161*, 143-149.

7. "Substrate Specificity and Inhibitors of a Capillary Injury-Related Protease (CIP) from Sheep Lung Lymph", Orlowski, M., Lesser, M., Ayala, J., Lasdun, A., Kam, C-M., and Powers, J. C. (1989) *Arch. Biochem. Biophys. 269,* 125-136.

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9. "Human Leukocyte and Porcine Pancreatic Elastase: X-ray Crystal Structures, Mechanism, Substrate Specificity, and Mechanism-Based Inhibitors", Bode, W., Meyer, E., and Powers, J. C. (1989) *Biochemistry 28,* 1951-1963.

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13. "Localization, Implications for Function, and Gene Expression of Chymotrypsin-like Proteinases of Cytotoxic RNK-16 Lymphocytes", Zunino, S. J., Allison, N. J., Kam, C-M., Powers, J. C., and Hudig, D. (1988) *Biochem. Biophys. Acta 967*, 331-340.

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